HISTOCHEMICAL STUDIES OF THE AUTONOMIC INNERVATION OF 'NORMAL' AND DISEASED HUMAN LUNG

Thesis submitted for the Degree of

MASTER
[DOCTOR] OF PHILOSOPHY

In the University of London

by

CATHERINE AMANDA SHIPPERBOTTOM BSc.

Department of Lung Pathology
Cardiothoracic Institute
Brompton Hospital
University of London

July 1988

ACKNOWLEDGEMENTS

During the time it has taken to prepare this thesis, many people have contributed their support and advice. I extend my warmest thanks to all of them.

Dr. P.K. Jeffery (Cardiothoracic Institute), my supervisor, from whom I have learned that all things are possible, given time.

Professor Corrin (Cardiothoracic Institute), for proof-reading and polite criticism of my spelling and grammar.

Dr's Johnston and Henry (University Hospital, Aberdeen) for generously providing archival material.

Dr. A. Morice (Cambridge University) for radioimmunoassay of tissue peptides.

Dr. P. Emson (Barabaham) for providing anti-substance P antibodies for radioimmunoassay.

Miss M. Rehan (Statistical advisor) for her patience.

The Asthma Research Council for financial support.

Mrs. S. Majundar (Cardiothoracic Institute) for her technical and moral support.

Mr. R. Godfrey and Mr B. Quinn (Cardiothoracic Institute) for reminding me I'm not on my own.

Special thanks to: Dr. D.F. Rogers (Cardiothoracic Institute), to whom I am indebted for his valuable advice,

Mr. R. Sharma (friend and colleague) for helping me 'keep my eye on the ball',

My family (including the Teakles) for their encouragement and support,

And Gavin, for believing in me and to whom this thesis is dedicated.

ABSTRACT.

Histochemical and immunohistochemical techniques are used to describe the relationships of the cholinergic, adrenergic and non-cholinergic, non-adrenergic nervous systems to effector structures in the airway mucosa of 'normal' and diseased human lung. The distribution of each type of nerve fibre is assessed by quantitative and semi-quantitative methods: i) at 5 airway levels in normal lung and ii) in disease. Samples of macroscopically 'normal' human airways were obtained from lungs resected for carcinoma. The disease groups comprise cystic fibrosis and asthma, where autonomic abnormality has been implicated in their pathogenesis. Disease control tissue was taken from bronchiectatic lungs and from a group of patients with various conditions including chronic bronchitis and emphysema. Examination of the normal airways stained for PGP 9.5, a marker of nervous tissue, revealed that the density of nerves decreases with each successive airway generation, with no staining beyond the level of the subsegmental bronchi. Both substance P and calcitonin gene-related peptide (CGRP) were present in intra-epithelial nerve fibres and CGRP was also present in neuroendocrine cells. Both were present in airway ganglia. They were, however, extremely sparse in association with bronchial smooth muscle and airway vasculature. Neither were present in submucosal gland. In contrast, large numbers of cholinergic and vasoactive intestinal peptide (VIP)-ergic nerves were found in the lamina propria, submucosal gland, bronchial smooth muscle and airway ganglia. VIP was more dense than the cholinergic supply around the blood vessels. Catecholaminergic nerves predominated around the airway vessels, particularly the bronchial, but elsewhere were generally more sparse than cholinergic or VIP-ergic nerves. Positive catecholamine fluorescence was present in submucosal glands and, to a lesser extent, bronchial smooth muscle and airway parasympathetic ganglia. Neuropeptide Y showed a similar distribution to the aminergic supply but was more frequently found in bronchial smooth muscle and was not present in gland. In disease, the bronchiectatic tissue showed a significant reduction in all types of neural marker in comparison with all the other groups. Except for VIPergic nerves, the CF tissue showed no difference in the density of neural markers in comparison with the young control group and normal tissue. The reduction in VIPergic nerves appeared to be inversely related to the degree of mucous gland hypertrophy. Compared with the normal tissue, the asthmatic tissue showed no marked differences in airway innervation.

It is concluded that a reduction in neural markers is likely to be secondary to the inflammatory changes due to infection, and that the reduction possibly contibutes to the symptoms of chronic obstructive lung disease.

CONTENTS.

Title page		1
Acknowlegements		2
Abstract		3
Contents		4
List of Figures		9
List of Plates		10
List of Tables		12
Chapter 1. INTRODUCTION.		15
1.1.	**The Autonomic Nervous System**	16
1.1.1.	Classical divisions	16
1.1.1.1.	The parasympathetic system	17
1.1.1.2.	The sympathetic system	19
1.1.2.	Recent advances: the 'NANC' system	22
1.1.2.1.	The concept of co-transmission	25
1.2.	**Autonomic Abnormality in Disease**	27
1.2.1.	Cystic fibrosis	28
1.2.1.1.	The search of the basic defect	30
1.2.2.	Asthma	33
1.2.2.1.	Autonomic abnormality in asthma	34
1.3.	**The Innervation of Normal Lung**	37
1.3.1.	Afferent pathways	37
1.3.2.	Efferent pathways	38
1.3.3.	Innervation of effector structures in the airway mucosa	41
1.3.3.1.	Surface epithelium	41
1.3.3.2.	Submucosal gland	42
1.3.3.3.	Bronchial smooth muscle	43
1.3.3.4.	Vasculature	45
1.3.3.5.	Airway ganglia	46
1.4.	**Techniques to Delineate Nerve Types**	48
1.4.1.	The parasympathetic system: Cholinesterase histochemistry	48
1.4.2.	The sympathetic system: Induced fluorescence of	

	catecholamines	51
1.4.3.	The NANC system: Immunohistochemistry	52
1.4.3.1.	Indirect immunofluorescence	53
1.4.3.2.	Avidin-Biotin enhancement	55
1.4.3.3.	Peroxidase, anti-peroxidase	55
1.4.3.4.	Immunogold/silver enhancement	57
1.4.4.	Electron microscopy	57
1.4.5.	Radioimmunoassay	58
1.4.6.	Spectrophotometry	58
1.5.	**Aim and objectives of the present study**	60
1.6.	**Summary of introduction**	62
Chapter 2. MATERIAL AND METHODS.		64
2.1.	**Patient Data**	65
2.2.	**Source of Tissue** and Airway Levels	66
2.2.1.	Fresh human lung	66
2.2.1.1.	Surgically resected tissue	66
2.2.1.2.	Transplanted lung tissue	66
2.2.1.3.	Post-mortem tissue	68
2.2.2.	Archival material (pre-embedded tissue)	68
2.2.3.	Control rat/guinea-pig tissue	68
2.3.	**Tissue Processing**	69
2.3.1.	'Snap' freezing	69
2.3.2.	Benzoquinone-fixed tissue	69
2.3.3.	Formalin-fixed tissue	69
2.3.4.	Archival material	70
2.4.	**Staining Techniques**	71
2.4.1.	Cholinesterase histochemistry	71
2.4.2.	Glyoxylic acid-induced fluorescence of catecholamines	73
2.4.2.1.	Pre-labelling experiments	75
2.4.3.	Immunohistochemistry	76

2.4.3.1. Indirect immunofluorescence 76
2.4.3.2. Avidin-Biotin enhancement 78
2.4.3.3. Peroxidase-anti-peroxidase 79
2.4.3.4. Immunogold/Silver enhancement 81
2.4.4. Radioimmunoassay 83
2.4.4.1. Assay of vasoactive intestinal peptide 83
2.4.4.2. Assay of substance P 85
2.4.5. Spectrophotometric determination of serum cholinesterase activity 85

2.5. Quantitative methods 88
2.5.1. Point counts 88
2.5.2. Methods for the evaluation of the number and density of nerve fibres in tissue sections 90
2.5.2.1. Quantitative assessments 90
2.5.2.2. Semi-quantitative assessments 94

2.6. Statistical Analysis 96
2.6.1. Levels of significance 96
2.6.2. Distribution of data and appropriate tests of significance 97
2.6.2.1. Point counts 97
2.6.2.2. Quantitative assessments 97
2.6.2.3. Semi-quantitative assessments 98
2.6.2.4. Correlation of data 99
2.6.2.5. Radioimmunoasssay 99
2.6.2.6. Serum cholinesterase activity 99

Chapter 3. RESULTS. 100

3.1. Qualitative assessments: normal lung 101
3.1.1. Overall distribution of nerve fibres: staining for PGP 9.5 and neuron-specific enolase 101
3.1.2. The distribution of substance P 103
3.1.3. The distribution of calcitonin gene-related peptide 104
3.1.4. Cholinesterase histochemistry: the distribution of cholinergic nerves 104

3.1.5.	The distribution of VIP-like immunoreactivity	105
3.1.6.	Catecholamine fluorescence	106
3.1.7.	The distribution of tyrosine hydroxylase: an alternative marker for aminergic nerves	107
3.1.8.	The distribution of neuropeptide Y (NPY)	108
3.2.	**Quantitative assessments: normal and diseased lung**	117
3.2.1.	Relative size of effector structures: point counts	117
3.2.2.	The quantification of epithelial nerves	119
3.2.3.	Quantification of substance P and CGRP-fibres	120
3.2.3.1.	Substance P	121
3.2.3.2.	CGRP	122
3.3.	**Semi-quantitative assessments: normal and diseased lung**	124
3.3.1.	Semi-quantitative assessment of the density of cholinergic nerves	124
3.3.1.1.	Results of the hierarchical analysis of variance	125
3.3.1.2.	Summary of cholinergic data	128
3.3.2.	Semi-quantitative assessment of the density of VIPergic innervation	129
3.3.2.1.	Analysis of variance; benzoquinone-fixed tissue	130
3.3.2.2.	Analysis of variance; formalin-fixed tissue	132
3.3.2.3.	Correlation of the score for VIP in submucosal gland with the % area of gland	136
3.3.2.4.	Summary of VIP data	140
3.3.3.	Semi-quantitative assessment of the density of aminergic nerves	142
3.3.3.1.	Results of the 2-way analysis of variance	143
3.3.3.2.	Summary of aminergic data	145
3.3.4.	Semi-quantitative assessment of the distribution of tyrosine hydroxylase-immunoreactivity	146
3.3.4.1.	2-way analysis of variance	
3.3.4.2.	Summary of tyrosine hydroxylase data	151
3.3.5..	Semi-quantitative assessment of the density of NPY	152
3.3.5.1.	Results of the 2-way analysis of variance	152
3.3.5.2.	Summary of NPY data	156

3.4.	**Comparative results**	157
3.4.1.	Results of Radioimmunoassay	157
3.4.1.1.	Amount of vasoactive intestinal peptide	157
3.4.1.2.	Amount of substance P	158
3.4.2.	Spectrophotometric determination of the activity of serum butyrylcholinesterase	158
3.5.	**Summary of Results**	160
Chapter 4. DISCUSSION.		165
4.1.	**The distribution of neural markers in normal lung**	166
4.1.1.	Epithelial innervation	166
4.1.2.	Is there a sparcity of substance P and CGRP in effector structures in human airways?	172
4.1.3.	Do substance P and CGRP have a 'motor' function in human lung?	174
4.1.4.	The control of tracheobronchial submucosal glands	176
4.1.5.	The control of bronchial smooth muscle	177
4.1.6.	The role of butyrylcholinesterase	182
4.1.7.	The control of airway vasculature	183
4.1.8.	Airway ganglia	186
4.1.9.	Are there differences in innervation at different airway levels?	190
4.2.	**The distribution of neural markers in disease**	196
4.2.1.	Cystic fibrosis	196
4.2.2.	Asthma	200
4.2.2.1.	The epithelium in asthma	200
4.2.2.2.	The bronchial smooth muscle in asthma	204
4.2.2.3.	The submucosal glands in asthma	208
4.3.	**Future Directions**	210
4.4.	**General Summary**	215
APPENDICES.		217
REFERENCES.		227

LIST OF FIGURES.

Chapter 1.

1.1. Mechanism of action of acetylcholine 20
1.2. Mechanism of action of noradrenaline 20
1.3. Manifestations of cystic fibrosis 28
1.4. Schematic diagram of parasympathetic and sympathetic pathways to the airway wall 40
1.5. Indirect immunofluorescence 54
1.6. Avidin-Biotin enhancement 54
1.7. Peroxidase-anti-peroxidase 54
1.8. Immunogold/Silver enhancement 54

Chapter 2.

2.1. Diagram of airway levels 67
2.2. Method of selection of high power fields for point counts 89
2.3. Method for incomplete sections 89
2.4. Method of counting nerve fibres within the epithelium 91
2.5. Wiebel grid 91

Chapter 3.

3.1. Relationship between the % area of submucosal gland per section and the score for VIP in different patient groups 138

Chapter 4.

4.1. Hypothetical diagram of efferent pathways to the airway wall 191
4.2. Hypothetical diagram of afferent pathways to the airway wall 192

LIST OF PLATES.

Plate 1. Cholinesterase Histochemistry 109

1. Cholinergic ganglion
2. Gland and smooth muscle
3. Gland acini
4. Small arterioles

Plate 2. Catecholamine Fluorescence 110

5. Adrenergic nerves in submucosal gland
6. Adrenergic nerves in bronchial smooth muscle
7. Parasympathetic ganglion

Plate 3. VIP immunofluorescence 111

8. VIP in submucosal gland
9. VIP in bronchial smooth muscle
10. VIP in normal gland
11. VIP in CF gland

Plate 4. Immunogold/Silver Enhancement 112

12. Substance P in epithelium
13. CGRP in neuroendocrine cell
14. PGP 9.5. in asthma epithelium

Plate 5. Immunogold/Silver Enhancement 113

15. PGP 9.5 in epithelium
16. PGP 9.5 in submucosal gland
17. CGRP in ganglion

Plate 6. Cholinesterase Histochemistry 114

18. Cholinergic nerve bundle in lamina propria
19. Butyrylcholinesterase in smooth muscle
20. Acetylcholinesterase in smooth muscle

Plate 7. Catecholamine Fluorescence 115

21. Adrenergic nerves around blood vessel
22. Long nerve fibre in bronchial smooth muscle
23. Small fibre in peripheral airway

Plate 8. Immunogold/Silver Enhancement 116

24. Tyrosine hydroxylase in bronchial smooth muscle
25. Tyrosine hydroxylase around arteriole
26. NPY in bronchial smooth muscle
27. NPY around arteriole
28. VIP in ganglion
29. VIP around arteriole

LIST OF TABLES.

Chapter 1.

Table

1.	Evidence of co-localisation of neurotransmitters	26

Chapter 2.

2.	Results of the dilution trial of anti-VIP antibody	77
3.	Results of the dilution trial of anti-substance P antibody	78
4.	Results of the dilution trial of anti-NSE antibody	80
5.	Dilution of primary antibody and controls for immunogold/silver enhancement	82

Chapter 3.

6.	Mean (+SEM) % areas of sections occupied by each effector structure	117
7.	Tissue used in the quantification of epithelial nerves	119
8.	Total epithelial length (mm) measured per group	119
9.	Mean (±SEM) number of nerves per mm length of epithelium in each group	119
10.	Tissue used in the quantification of substance P and CGRP-immunoreactivity	120
11.	Mean (±SEM) number of substance P fibres per section	121
12.	Mean (±SEM) number of CGRP fibres per section	122
13.	Tissue used in the assessment of the density of cholinergic nerves	125
14.	Mean score (+SEM) of the density of cholinergic nerves in normal and CF tissue	126
15.	Mean score (+SEM) of the density of cholinergic nerves at different airway levels in normal lung	126
16.	Cholinergic nerves: hierarchical analysis of variance of the density in normal and CF lung	127

17.	Benzoquinone-fixed tissue used in the assessment of the density of VIP-immunoreactivity	129
18.	Formalin-fixed, paraffin-embedded tissue used in the assessment of the density of VIP-immunoreactivity	129
19.	Mean scores (+SEM) of the density of VIP-immuno-reactivity in benzoquinone-fixed normal and CF tissue	130
20.	Mean scores (+SEM) of the density of VIP-immuno-reactivity at different airway levels in normal lung	130
21.	VIPergic nerves: hierarchical analysis of variance of the density in benzoquinone-fixed normal and CF tissue	131
22.	Mean scores (+SEM) of the density of VIP-imumuno-reactivity in formalin-fixed, paraffin embedded tissue	132
23.	VIPergic nerves: 2-way analysis of variance of the density in normal and CF tissue	133
24.	VIPergic nerves: 2-way analysis of variance of the density in normal and bronchiectatic tissue	134
25.	VIPergic nerves: 2-way analysis of variance of the density in normal and 'young' tissue	135
26.	VIPergic nerves: 2-way analysis of variance of the density in 'young' and CF tissue	136
27.	% area of submucosal gland per tissue section and the corresponding score for VIP	137
28.	Spearman's rank correlation coefficient for the % area of submucosal gland and the score for VIP	137
29.	Tissue used in the assessment of the density of aminergic nerves	142
30.	Mean scores (+SEM) of the density of aminergic nerves in normal lung	143
31.	Aminergic nerves: hierarchical analysis of variance of the density in normal lung	144
32.	Tissue used in the assessment of the density of tyrosine hydroxylase-immunoreactivity	146
33.	Mean scores (+SEM) of the density of tyrosine hydroxylase-immunoractivity	147
34.	Tyrosine hydroxyalse: 2-way analysis of variance of the density in normal and CF tissue	148

35. Tyrosine hydroxylase: 2-way analysis of variance of the density in normal and bronchiectatic tissue 149
36. Tyrosine hydroxylase: 2-way analysis of variance of the density in normal and 'young' tissue 150
37. Tissue used in the assessment of the density of NPY-immunoreactivity 152
38. Mean scores (+SEM) of the density of NPY-immuno-reactivity 152
39. NPY-immunoreactivity: 2-way analysis of variance of the density in normal and CF tissue 153
40. NPY-immunoreactivity: 2-way analysis of variance of the density in normal and bronchiectatic tissue 154
41. NPY-immunoreactivity: 2-way analysis of variance of the density in normal and 'young' tissue 155
42. NPY-immunoreactivity: 2-way analysis of variance of the density in 'young' and CF tissue 156
43. The amount of VIP in samples of normal and CF lung.
44. The amount of substance P in samples of normal and CF lung 158
45. Butyrylcholinesterase activity in samples of human serum 159

CHAPTER 1.

INTRODUCTION

The role of the autonomic nervous system (ANS) in the lung and other organs has recently been the subject of intense research investigation and it has been shown that the nervous control of the airways is more complex than originally supposed. The concept of a non-adrenergic, non-cholinergic system being part of the ANS is now widely accepted and there is evidence that neuropeptides act as the neurotransmitters. Several neuropeptides have recently been identified in human airways and although they have potent effects, their pathophysiological role is uncertain. Autonomic abnormality has been implicated in several disorders of man, including asthma and cystic fibrosis and an understanding of the autonomic nervous system is essential to our understanding of these diseases. The studies herein describe the different autonomic nerves and neuropeptides in normal human lung, and in lung from patients with cystic fibrosis and asthma.

1.1 THE AUTONOMIC NERVOUS SYSTEM.

1.1.1 Classical divisions.

The autonomic nervous system has, until recently, been considered to consist of two major divisions; the parasympathetic (craniosacral) and sympathetic (thoracolumbar) divisions. In 1904, Elliot demonstrated that an extract of the adrenal gland, adrenaline, mimicked the action of the sympathetic nerves, but it was not until 1921 that the concept of chemical neurotransmission gained acceptance. This stemmed largely from the experimemts of Otto Loewi who demonstrated that a chemical substance released from the vagus nerve caused slowing of the beat of isolated frog heart. It was later shown that this substance was acetylcholine. Gradually, support for acetylcholine acting as a chemical neurotransmitter extended to other autonomic synapses when it was shown that presynaptic neurons liberate acetylcholine in

sympathetic ganglia [Feldberg 1945]. Noradrenaline was subsequently shown to be the post-ganglionic transmitter at the effector structure [Von Euler 1946].

A general feature of the autonomic nervous system is that, unlike skeletal neuromuscular junctions, only occasional membranous contact is established between nerve fibres and target cells. The neuro-effector junction in the autonomic nervous system is characterised by areas of thickened membrane on terminal nerve varicosities and there is no apparent post-junctional specialisation. The majority of axons lie at distances of about 0.1um from the target cell. The transmitters therefore reach the target cell by diffusion [Rhodin 1973].

Receptors for the neurotransmitter are present on the target cell. These are classified according to their location and subsequent action when stimulated by the neurotransmitter. The receptors on the target cell are subject to a negative feedback mechanism whereby prolonged exposure of the receptor to relatively high concentrations of the neurotransmitter leads to a decrease in the binding affinity of the receptor for the transmitter and a consequent reduction in biological effect [Katz and Thesleff 1957, Lefkowitz 1978].

1.1.1.1 The Parasympathetic system.

It is now well established that in the parasympathetic system, nearly all the pre-ganglionic and post-ganglionic fibres are cholinergic (i.e. acetylcholine is the neurotransmitter). The acetylcholine is synthesised in the nerve cell bodies and stored in vesicles in the nerve endings. The enzyme involved in the final stage of production of acetylcholine is choline acetyltransferase. The acetylcholine is released from the vesicles on the arrival of a nerve

impulse at the synapse. Calcium ions are necessary for its release. The vesicles, as visualised by electron microscopy, are agranular and about 30-60nm diameter [Richardson 1966]. The enzyme acetylcholinesterase is responsible for the rapid hydrolysis of the acetylcholine to choline and acetic acid The reaction takes place in the synaptic cleft and controls the amount of acetylcholine that reaches the target cell. The role of butyryl-cholinesterase or pseudocholinesterase in this context is unknown. Butyrylcholinesterase will hydrolyse acetylcholine [Myers 1953] and unlike acetylcholinesterase, is not inhibited by high concentrations of acetylcholine. However, it is not known whether this affects nervous transmission.

The receptors for acetylcholine were initially described as being of two basic types; muscarinic and nicotinic [Dale 1914]. These have since been further classified:-

a. Muscarinic

type 1 - preganglionic; high affinity for pirenzipine

type 2 - postganglionic (on nerve); low affinity for pirenzipine [Hammer and Giachetti 1982]

type 3 - not yet fully characterized; postulated on effector organ [Birdsall and Hulme 1983]

b. Nicotinic - present on ganglia in the central nervous system and directly on skeletal muscle [Lummis and Satelle 1985, Clarke 1985]. There may be more than one type [Zygmunt et al 1983].

The activation of these receptors sets up a biochemical cascade or chain reaction in the target cell. In the case of the cholinergic receptors, activation produces an increase in cellular uptake and release of calcium ions which activates phospholipid metabolism [see fig.1.1]

Physiological studies on isolated human airways demonstrate bronchoconstriction in response to electrical field stimulation. This response is blocked by atropine, demonstrating cholinergic control of the smooth muscle, and abolished by tetrodotoxin, a sodium channel blocker, showing the effect to be nerve mediated [Richardson and Beland 1976, Davis et al 1982, Taylor 1984].

Vagal stimulation causes an increase in the output of mucus from the bronchial submucosal glands of animals both in vivo [Davis et al 1976] and in vitro [Borson et al 1974]. Studies of isolated human bronchial mucosa show that electrical field stimulation of cholinergic nerves causes an increase in the output of mucous that is blocked by both atropine and tetrodotoxin. These secretory effects are thought to be mediated by cholinergic muscarinic receptors [Ukei et al 1980].

1.1.1.2. The sympathetic system.

In the sympathetic system, the pre-ganglionic transmitter is acetylcholine and the post-ganglionic nerves are mostly noradrenergic. Other biogenic amines have since been implicated in neurotransmission e.g. dopamine and serotonin [Hornykiewicz 1973, Cottrell and Macon 1974]. Sympathetic nerve terminals contain granular vesicles of about 30-60nm diameter that are thought to contain the catecholamine neurotransmitter [Richardson 1966]. Noradrenaline is released upon electrical stimulation of the nerve; it is subsequently recaptured by the nerve terminal and recycled [Iverson 1967].

There are two major types of adrenergic receptors, alpha and beta [Alquist 1948]. Alpha receptors are located mainly in the radial muscle of the iris, arterioles, veins, the sphincters of the stomach and small intestine, urinary bladder and the pilomotor muscles and sweat glands of the skin, whereas

Fig.1.1 Mechanism of action of acetylcholine

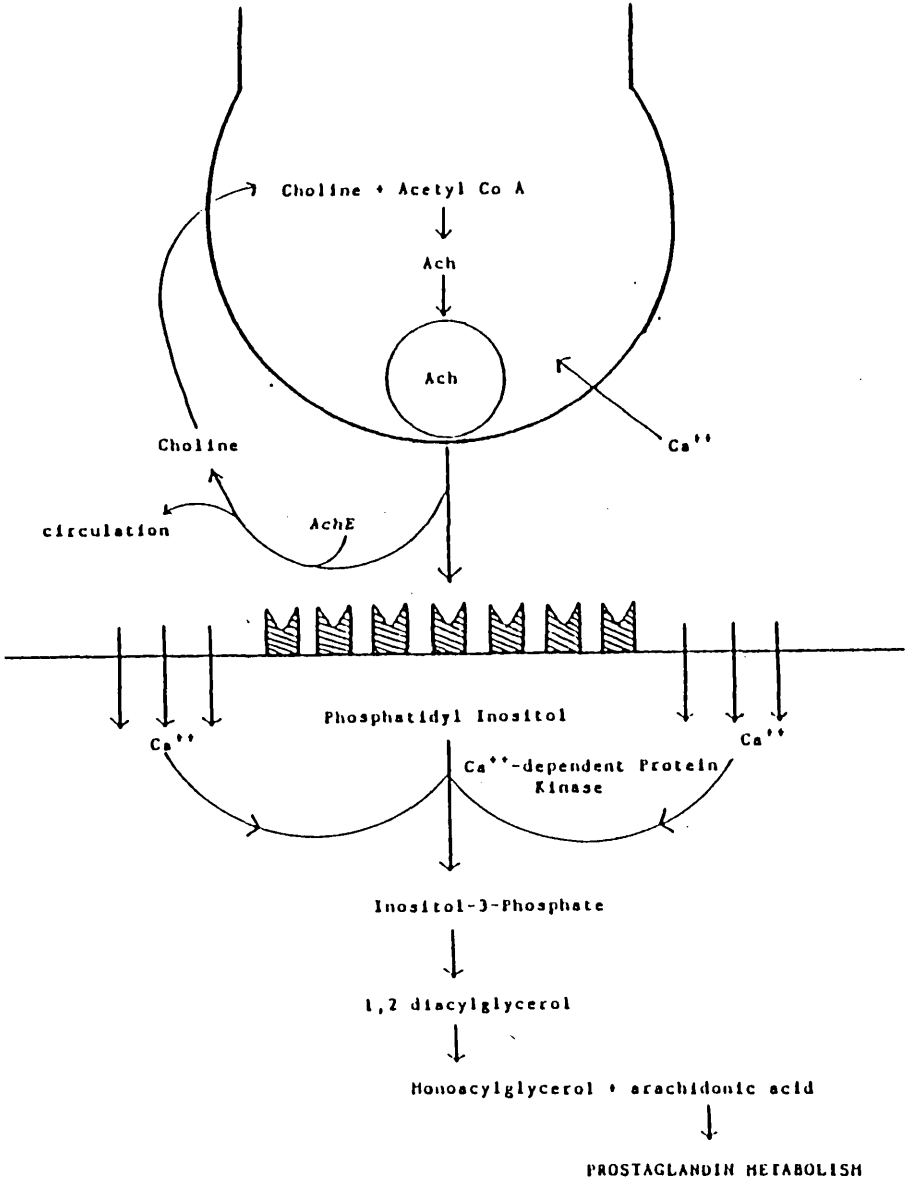
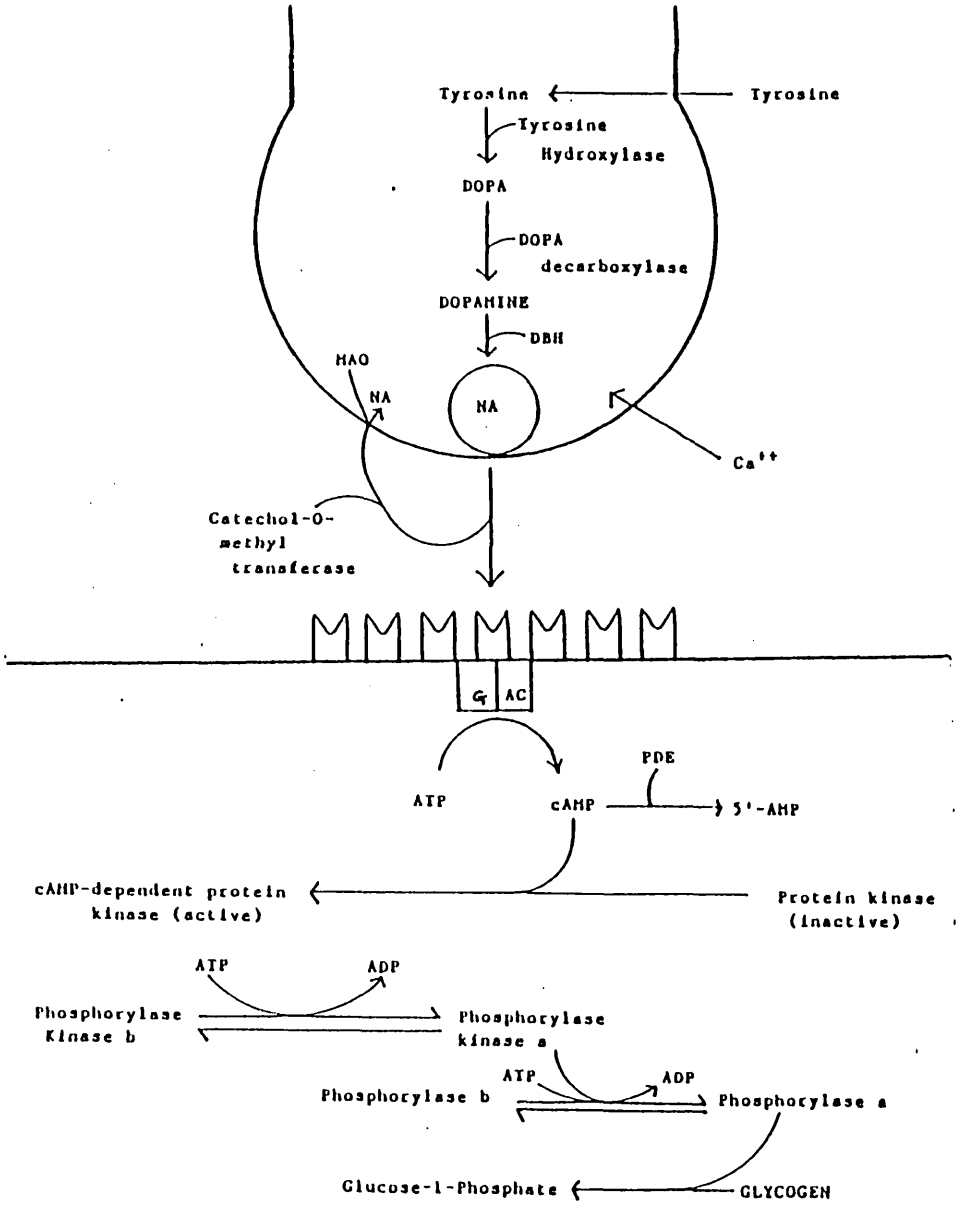


Fig.1.2 Mechanism of action of noradrenaline



beta receptors are found in the heart and in bronchial smooth muscle. Both types are present on skeletal muscle arterioles. Alpha and beta receptors have been further classified into two types [Langer 1974, Berthelson and Pettinger 1977, Nahorski 1978]. $Beta_1$ receptors are mainly located on cardiac muscle and type two elsewhere in the body, including bronchial smooth muscle.

Activation of beta receptors stimulates the formation of cyclic AMP via adenylate cyclase: activation of alpha receptors acts through the calcium 'second messenger system' [see fig.1.2].

Functional studies in guinea-pigs have demonstrated neurally mediated relaxation of airway smooth muscle which is blocked by β-antagonists in the trachea but not bronchi [Zaagsma 1983]. Such a finding is consistent with the pattern of innervation in this species. In vitro studies on cholinergically precontracted human airways show a propanalol independent but tetrodotoxin-sensitive relaxation of the smooth muscle, suggesting a lack of functional sympathetic innervation [Richardson and Beland 1976, Doidge and Satchell 1982]. Also, inhaled β-adrenoceptor agonists have no effect on resting bronchomotor tone in normal persons, indicating an absence of tonic resting bronchodilation [Richardson and Stirling 1969, Tattersfield 1973].

Sympathetic stimulation also promotes mucus secretion [Gallager 1975, Ukei 1980] which is abolished by both ∝-adrenoceptor blockade [Borson et al 1980, Peatfield and Richardson 1982] and β-antagonists [Phipps et al 1982].

Adrenergic nerves usually mediate vascular smooth muscle contraction via ∝-receptors [Burnstock 1983] although the role of sympathetic control of blood flow to the tracheobronchial tree has not been studied. Auto-radiographical mapping has demonstrated few ∝-adrenoceptors in large airways [Kneussel and Richardson 1978, Black et al 1981]

1.1.2. Recent advances: the 'non-adrenergic, non-cholinergic' system.

More recently, physiological studies have indicated a third component of the autonomic nervous system which has been termed the 'Non-Adrenergic, Non-Cholinergic' (NANC) system [Burnstock 1963]. The first indication of the existence of this system came from the work of Langley and colleagues at the end of the nineteenth century [Langley and Anderson 1895]. They described an atropine-resistant excitation of the bladder in response to pelvic nerve stimulation. Later, a similar phenomenon was reported in the stomach where inhibition was observed on vagal stimulation when the cholinergic excitatory nerves had been blocked with atropine [Langley 1898]. It was not until the 1960s, however, that evidence was presented of nerves in the gut and the bladder belonging to a group that were neither adrenergic nor cholinergic [Burnstock et al 1963, Martinson and Muren 1963, Martinson 1965]. Since then NANC nerves have been described in the gastrointestinal tract and the urogenital, cardiovascular and respiratory systems of mammals [see Burnstock 1969].

The nature of the transmitters of these nerves is still a subject of controversy and many substances have been proposed. In 1972 Burnstock formulated the 'purinergic' nerve hypothesis which was based on observations of the actions of adenosine triphosphate (ATP), which relaxes intestinal smooth muscle and contracts bladder. Considerable evidence has since accumulated in support of this hypothesis. An important advance was the identification of purino-receptors: type P-1 which bind adenosine and are blocked by methylxanthines and P-2 which bind ATP and are methylxanthine resistant [Daly 1982]. Although the evidence for purinergic neurotransmission is strong in gut and bladder, it seems likely that other substances may mediate the NANC responses in other organs. The development of

immunohistochemical techniques to localise various biologically active polypeptides [Holkfelt et al 1980] added to the increasing evidence for the 'peptidergic' nerve theory. Several peptides have been localised in autonomic nerve terminals and proposed as neurotransmitters. These include vasoactive intestinal peptide (VIP), a 28 amino-acid peptide, substance P, an undecapeptide with C-terminal end sequence homologies with other mammalian peptides (collectively termed 'tachykinins'), calcitonin gene-related peptide (CGRP), a 37 amino-acid peptide and neuropeptide Y (NPY), a 36 amino-acid peptide with tyrosine at the N-terminus.

NANC innervation in the lower respiratory tract was first described in guinea-pig trachea [Coburn and Tomita 1973]. Electrical field stimulation selectively stimulated the intrinsic nerves to make smooth muscle contract. The contractile response due to atropine and the relaxation pathway due to adrenergic nerve activity was stimulated by adrenergic agonists. However, only a partial relaxation was achieved, indicating the presence of a non-adrenergic inhibitory pathway. Application of tetrodotoxin abolished the response, demonstrating it to be nerve-mediated [Coburn and Tomita 1973, Coleman and Levy 1974]. NANC nerves have since been described in human airways [Richardson 1981, Barnes 1984].

Exogenously applied adenosine will relax precontracted guinea-pig tracheal smooth muscle [Coleman 1976, Satchell 1980], but the purinoceptor agonists theophylline and quinidine are ineffective at blocking this inhibitory NANC response [Coleman 1980, Grundstrom et al 1981]. Also, the characteristics of the relaxation response differ from those produced by electrical field stimulation [Karlsson and Persson 1984] indicating that purines are less likely to be the mediators of NANC relaxation responses in the lung.

Several neuropeptides have been localised by immunocytochemistry to nerves and neuroendocrine cells in mammalian airways [see Polak and Bloom 1986].

Of the several peptides isolated from human airways only VIP and a related peptide, peptide histidine methionine (PHM) have been shown to initiate smooth muscle relaxation [Palmer et al 1986a]. VIP was originally isolated from gut [Said and Mutt 1970] and there is now convincing evidence that VIP acts as the neurotransmitter in the non-adrenergic relaxation pathway in the lung [Richardson and Beland 1976, Doidge and Satchell 1982], although in the absence of specific antagonists for VIP receptors it is difficult to prove this conclusively. VIP has been shown to be released on stimulation of NANC nerves in the guinea-pig trachea, and incubation of the lung tissue with an antibody to VIP reduces NANC responses [Cameron et al 1983]. As well as producing potent bronchodilatory effects VIP is also a strong vasodilator [Malm et al 1980] and stimulates electrolyte transport across bronchial mucosa [Nathanson et al 1983]. VIP receptors have been described [Taton et al 1981] and localised by autoradiography in human lung [Carstairs and Barnes 1986]. VIP receptors have been shown to be coupled to adenylate cyclase [Robberecht et al 1981].

Other peptides localised to efferent nerves in the mammalian respiratory tract include neuropeptide Y [Sheppard et al 1984b, Uddman et al 1984] which has been shown to have profound effects on blood flow and seems to be co-localised with noradrenaline [Lundberg 1982].

Excitatory pathways independent of cholinergic stimulation have also been described in animal airways [Andersson and Grundstrom 1983]. There is evidence that substance P and CGRP may be the neurotransmitters. Both substance P and CGRP are potent bronchoconstrictors [Nilsson et al 1977, Palmer et al 1986] and they may be co-localised with one another [Lundberg et al 1985a]. Substance P appears to be localised in afferent nerve terminals: treatment with the sensory nerve poison, capsaicin, depletes sensory nerve terminals of substance P [Lundberg et al 1983a]. Binding of

substance P to specific receptors appears to mobilise intracellular calcium ions resulting in receptor-mediated phosphoinositide breakdown [Watson and Downs 1983, Holzer and Lippe 1984].

Several neuropeptides have also been localised to neuroendocrine cells, which can be considered to be part of the sensory systems in the lung [Pack and Widdicombe 1984]. These include bombesin, calcitonin, leucine-enkephalin and CGRP [Cutz 1981].

1.1.2.1. The concept of co-transmission.

The discovery that nerve terminals contain more than one type of vesicle [Cook and Burnstock 1976] led to the suggestion that individual nerves may potentially release more than one transmitter [Burnstock 1976]. The concept of co-transmission has since gained much support [Cuello 1982, Osborne 1983]. The substances released from the nerve may act as co-transmitters, each occupying its own receptors on the effector cell, or as neuromodulators, modifying the release or action of the neurotransmitter. The evidence for various cases of co-localisation of two or more transmitters within the same nerve is summarised in Table 1.

TABLE 1.

Evidence of co-localisation of neurotransmitters.

Transmitter	Location	Authors
Noradrenaline (NA) + ATP	vas deferens	Westfallet al 1978.
NA + Acetylcholine + purine	cultured sympathetic nerves	Potter et al 1983
NPY + NA	sympathetic nerves	Lundberg et al 1982, 1985b
ATP + SP	sensory reflex	Burnstock 1977
VIP + Acetylcholine	salivary gland	Bloom and Edwards 1980
SP + 5-HT	myenteric plexus	Legay et al 1984
CGRP + SP	lower airways & heart	Lundberg et al 1985a.

1.2. AUTONOMIC ABNORMALITY IN DISEASE

> "It is more probable that our ideas concerning the behaviour of the autonomic nervous system in man, and especially in disease, are elementary"
>
> H.L. Alexander 1933.

55 years on, our understanding of the role of the autonomic nervous system in human diseases has improved very little. However, autonomic abnormality has been shown to underlie certain disorders, including Homer's syndrome, the Riley-Day syndrome, hyperhidrosis and hypothalamic dysfunction [Walton 1982], Hirchsprung's disease [Bishop et al 1980a], oesophageal achalasia [Gridelli et al 1982] and Crohn's disease [Bishop et al 1980b]. Two common disorders of the lung in which autonomic dysfunction has been implicated are asthma and cystic fibrosis. However, the basic defect in either of these conditions remains unknown. Several theories have emerged but in vitro studies have been handicapped by a lack of fresh tissue and the inadequacy of the few animal models that have been proposed. The role of the NANC system in disease is therefore largely a matter for speculation. Disturbances in the NANC innervation of the gut have been shown to underlie Hirchsprung's disease and Crohn's disease [Bishop et al 1980a, 1980b] but the possible consequences of a similar dysfunction in the lung remain unkown. Changes in the autonomic nerves of the airways in disease have not been studied. The present study will attempt to identify any changes in the autonomic nerve supply to the lung that may occur in asthma or cystic fibrosis.

1.2.1. Cystic fibrosis.

Cystic fibrosis has been recognised since the late 1930's [Anderson 1938]. It is the most common inherited disorder and the most frequent cause of chronic suppurative lung disease in caucasian children, occurring in about 1:2000 live births each year [Hall and Simpkiss 1968, Conneally et al 1973]. It is inherited in Mendelian fashion as an autosomal recessive trait [Rocholz 1957] and is present as a single mutant allele [Danks et al 1965]. With the expansion of techniques for examination of chromosomes and gene mapping much effort has been concentrated on the search for the genetic defect.

The primary defect is related to abnormalities of exocrine gland secretion. The pathological changes seen in cystic fibrosis are summarised in fig. 1.3

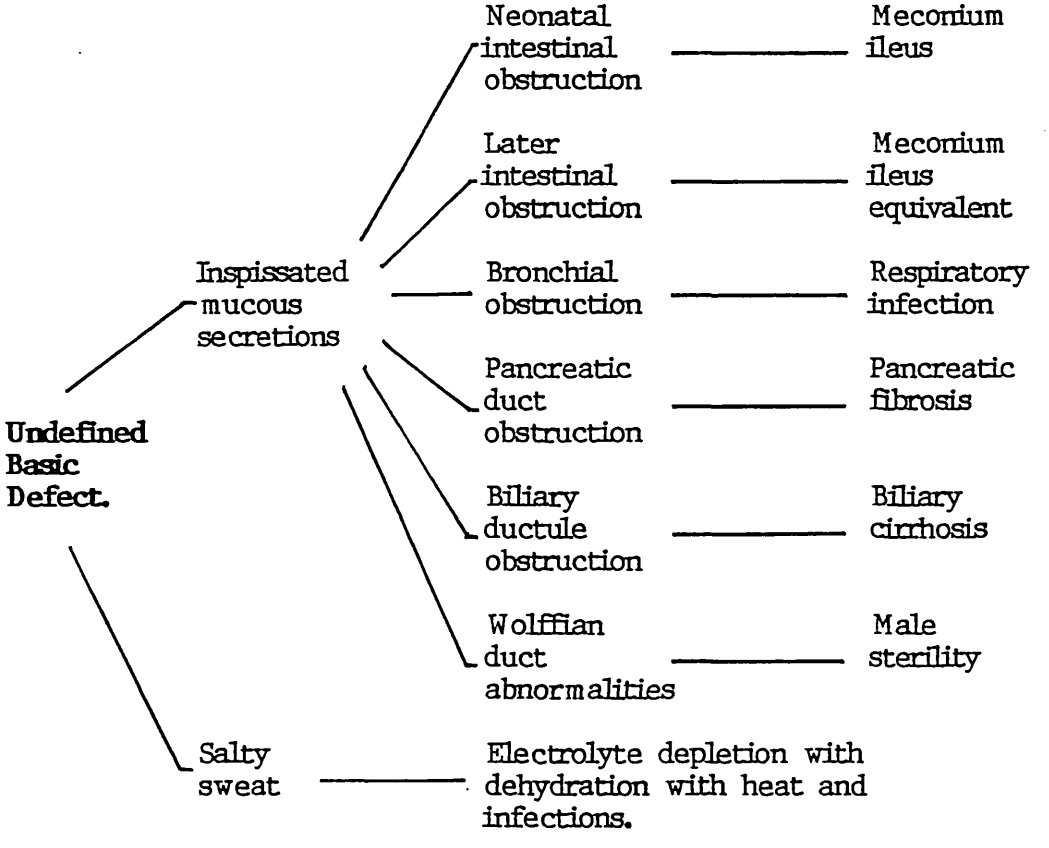


Fig.1.3 The manifestations of cystic fibrosis.

In the lung both upper and lower respiratory tracts are involved. Dilation of the mucous glands and ducts is the earliest feature [Anderson 1962]. However the glands are histologically normal at birth and the pathological changes are thought to be due to a secondary obstruction/infection [Oppenheimer and Esterly 1973, Oppenheimer 1981]. The lungs are unable to clear the thick viscid secretions (hence the alternative name, mucoviscidiosis) and the bronchioles and bronchi gradually fill up. Defective muco-ciliary clearance [Yeates et al 1976] and subsequent inability to keep the airways free of potential pathogens leads to colonisation of the obstructed airways by Pseudomonas aeruginosa of mucoid strain, Staphylococcus aureus and Haemophilus influenzae. Areas of scattered atelectasis develop, alternating with areas of emphysema. Infection gradually destroys the airway wall and extends into the peribronchial tissues leading to peribronchial fibrosis and bronchiectasis. Destruction of the bronchial wall may lead to the formation of abcesses. The airways obstruction from retained bronchial secretions and progressive peribronchial fibrosis eventually leads to impaired O_2/CO_2 exchange. Death is due to infection, respiratory failure or right heart failure. At post mortem, examination of the bronchial glands reveals distension, blockage of ducts and obstructive cellular damage distal to the blockage [Zueizer and Newton 1949, Oppenheimer 1981]. There appears to be an abnormal ratio of serous to mucous cells in CF but no qualitative changes are apparent in the serous component [Lamb and Reid 1972]. The sulphate content of CF mucins may be increased but the significance of this is unclear [Reid and deHaller 1967].

In addition to these histological observations there are abnormalities of ion and water content of cells and secretions. Sweat sodium and chloride are elevated [Schwachman and Mohmoodian 1979, Dearborn 1976]. These observations have led to various studies on gland and secretions in order to

define a physical or chemical abnormality, and to searches for humoral factors, which, if present in the circulation, could explain the widespread manifestations of the disease.

1.2.1.1. The search for the basic defect.

i. Humoral factors.

'CF specific factors' were first described by Spock in 1967 and they have since been reviewed by Wilson [Wilson 1983]. A factor derived from CF serum was shown to disorganise the beat of animal ciliated epithelium [Spock et al 1967]. However the observation that normal lung retains a normal potential difference when transplanted into a patient with cystic fibrosis [Alton et al 1987] has cast doubt on the role played by these CF factors.

ii. Channel activity/control.

The demonstration that the increased concentration of chloride ions in the sweat of patients with CF is due to a chloride reabsorption abnormality in the duct of sweat gland (up to 10% more impermeable) was an important advance [Quinton 1982, Quinton and Bijman 1983]. In patch clamp studies on cultured human airway epithelium, CF cell Cl^- channels have been shown to be biophysically identical to normal Cl^- channels i.e. they function as normal Cl^- channels when separated from the cell [Welsh and Liedtke 1986, Frizzell et al 1986]. However the pathway of cAMP induced activation of Cl^- channel activity, which is normally elicited by B-adrenergic receptor activation, is defective; there is no stimulation of channel activity in intact CF cells with noradrenaline or cAMP [Frizzell et al 1986]: cultured CF leucocyte populations show less of an increase in cAMP in response to isoproterenol than normal leucocytes [Davis et al 1978] and in lymphocyte

and granulocyte preparations from CF patients, although basal and prostaglandin E_1-stimulated levels of cAMP are normal, in response to the β-agonist, isoproterenol, cAMP is markedly reduced [Galant et al 1981]. Application of isoproterenol fails to elicit a response in either sweat gland or preparations of airway epithelium or salivary gland from CF patients, even though normal increases in cAMP are found [Sato and Sato 1984, McPherson et al 1985, Welsh and Leidtke 1986]. In response to cholinergic agonists, (which is mediated by Ca^{++} dependent processes) isolated eccrine sweat glands show a normal increase in sweat secretion indicating that it is the pathways controlling cAMP-mediated channel opening that are defective in CF. Reduced β-receptor numbers on whole neutrophils and lung homogenates from CF patients have been reported [Galant et al 1981, Sharma and Jeffery 1986], but β-receptor numbers appear similar to normal patients on lymphocytes from CF patients [Davis et al 1982]. Plasma noradrenaline levels are similar in the blood of patients with and without CF where the CF leucocytes displayed insensitivity to isoproterenol [Lake et al 1979], indicating that the insensitivity is not due to desensitisation of the receptors, but may be due to damage of the receptors in disease. It is not known whether damage occurs to the nerve supply of the lung in CF.

iii. Autonomic abnormalities in CF.

Autonomic abnormality in cystic fibrosis has long been proposed [Henderson et al 1979, Davis and Kaliner 1983]. The secretory activity of sweat and other glands is controlled by adrenergic and cholinergic innervation [Sato 1977] and by the NANC system [Holkfelt et al 1980]. Patients with CF have an exaggerated response to cholinergic agonists which is greater than that seen in normals [Sibinga 1961, Davis et al 1980]. 50% of CF sufferers display methacholine sensitivity and 25% show abnormal responses to inhaled

histamine. The methacholine hyperreactivity correlates with the severity of the pulmonary disease [Mitchell et al 1978]. However, patients with CF and their parents show increased sensitivity to the cholinergic agonist carbamylcholine; subjects included with atopy or pancreatic insufficiency did not account for the differences observed. [Davis et al 1980] indicating that the defect may be primary to the disease.

Other autonomic abnormalities oberved in patients with CF include abnormal blood pressure [Lieberman and Rodbard 1975], increased rates of sweating [Sibinga 1961] and slower pupillary dilation in response to darkness and painful stimuli [Rubin et al 1966], although the last mentioned may be due to pancreatic insufficiency. Submucosal gland discharge is also increased to a greater degree than that seen in normal gland following application of cholinergic agonists and is less inhibited by antagonists [Sturgess and Reid 1972a]. Hypertrophied glands including those from patients with CF show an augmented response to acetylcholine and are less easily inhibited by atropine [Sturgess and Reid 1972a]. Adrenergic drugs however, have no effect on the cycle of mucin synthesis and secretion which in human gland takes approx 4 hrs [Sturgess and Reid 1972a,1972b].

There is no good animal model of CF. Progress in understanding the disease has come from observations on cultured cells and from histological studies of post-mortem tissue or biopsy samples. One such study, utilising skin biopsies taken from patients with CF has demonstrated an absence or paucity of VIP-like immunoreactivity around CF sweat glands [Heinz-Erian et al 1985]. In view of the recent emergence of VIP as a putative, autonomic neurotransmitter, these findings may be of primary importance in the pathology of the disease. Abnormalities in the VIPergic innervation of the gut have shown the involvement of VIP in the pathology of several diseases including

Hirchsprung's disease [Bishop et al 1980], Crohn's disease [Bishop et al 1980b] and the Verner-Morrison syndrome [Sagor et al 1982].

Possible changes in the distribution of autonomic nerves to the lung in CF is an aspect of the disease which has recieved little attention. The present study examines the distribution of VIPergic nerves in CF lung, as well as that of other neuropeptides (CGRP, SP and NPY), in addition to the distribution of the 'classical' neurotransmitters, noradrenaline and acetylcholine.

1.2.2. Asthma.

'Asthma can be defined as a reversible increase in airways resistance, associated with mechanical obstruction to the flow of air and hyperreactivity of the airways to irritant stimuli' [Skidmore and Vardey 1984]. The condition is typified by an increased responsiveness of the tracheobronchial tree to various stimuli e.g. inhaled allergens, histamine aerosol, cold air etc. Asthma is usually classified as extrinsic or intrinsic, depending on whether or not there is a clearly defined precipitating cause.

Fatal asthma is characterised by plugging of the small airways (<2mm diameter) with thick tenacious mucus, oedema of the bronchial mucosa and infiltration by plasma cells, lymphocytes and eosinophils. The basement membrane of the bronchial epithelium is thickened and there is desquamation of the epithelium with consequent loss of ciliary action. The airway muscle is hypertrophied. All these changes may occur in non-fatal, mild asthma but there are few such histological studies due to the lack of available tissue.

1.2.2.1. Autonomic abnormality in asthma.

Eppinger and Hess [1909] were among the first to suggest that asthma was the result of increased vagal tone. Autonomic abnormality is common to all asthma sufferers and asthma can be regarded today as a complex condition involving the interplay of both immunological and neural factors. For example, inflammatory cell mediators influence autonomic activity [Kaliner et al 1972]. Several theories of autonomic imbalance have been proposed, as follows:-

i. Cholinergic mechanisms in asthma.

Enhanced cholinergic activity of bronchial smooth muscle has been suggested by several authors as a possible cause of bronchial smooth muscle hyper-reactivity [Simonssson et al 1967, Reed 1974]. Several mechanisms could account for this defect:

a. Facilitation of neurotransmission through parasympathetic ganglia and efferent nerves by inflammatory mediators [Hahn et al 1978, Scheller et al 1982].

b. Increased-end organ responsiveness to acetylcholine e.g. by increased numbers of muscarinic receptors or a post-receptor secondary messenger defect, or a dysfunction of cholinesterase. A defect in the action of acetylcholinesterase in asthma leading to increased levels of acetylcholine could account for the increased bronchial tone, although normal levels of the enzyme and of acetylcholine are present in asthmatic blood [Scudamore et al 1954]. The present study will examine the levels of butyrylcholinesterase, the role of which is unknown, in normal and asthmatic serum.

ii. Adrenergic mechanisms in asthma.

Enhanced ∝-adrenergic [Reed 1974, Szentvanyi 1968] or reduced B-adrenergic activity [Reed 1974, Szentvanyi 1968] could also account for an increase in bronchial smooth muscle activity. There is an apparent lack of a functional sympathetic innervation of human airway smooth muscle, which of itself is richly endowed with adrenergic β_2-receptors [Zaagsma et al 1983, Carstairs et al 1985]. There is much controversy about the existence of adrenergic nerve fibres in human airway smooth muscle [Laitenen 1985a, Sheppard et al 1983, Partanen et al 1982]. Catecholamines may also have effects on airway parasympathetic ganglia. This hypothesis is supported by findings of adrenergic nerve fibres in human airway ganglia [Richardson and Ferguson 1979]. The presence of an adrenergic component in human bronchial smooth muscle and airway ganglia is examined in the present study.

The role of ∝-receptors in asthma seems to be limited. There are few alpha receptors present on human airway bronchial smooth muscle [Phillips et al 1983]. Inhaled clonidine, an alpha-2 agonist, has been shown to inhibit a vagally induced increase in tracheal pressure [Anderson et al 1985] and may act by selectively turning off axon reflexes.

iii. NANC mechanisms in asthma.

Enhanced non-cholinergic excitatory activity [Lundberg et al 1983b] or non-adrenergic inhibitory mechanisms of bronchial smooth muscle activity [Richardson 1981, Barnes 1984] have been proposed to explain the hyperreactivity observed in asthma. However, as yet there have been few physiological and no histochemical studies on human asthmatic tissue either in vivo or in vitro.

The following hypotheses have been advanced to explain some of the symptoms of asthma:-

a. Blocked relaxation response. A hypothetical dysfunction in the NANC system could occur as a result of the inflammatory response. Mediators released from mast cells, neutrophils and eosinophils could break down VIP (which is susceptible to enzymatic attack) and therefore block the relaxation response induced by VIP, leading to increased cholinergic tone [Barnes 1987].

b. Increased contractile response. An alternative explanation is that in asthma there is an increase in airway afferent receptor discharge, perhaps due to inflammation e.g. by release of histamine, leukotrines or prostaglandins [Fuller et al 1986a, Fuller et al 1986b] or by the exposure during epithelial sloughing of afferent nerve endings below and within the epithelium. Increased substance P activity could account for many of the pathological changes seen in asthma including contraction of the airway smooth muscle [Karlsson et al 1984], extravasation of plasma and airway wall oedema [Lundberg et al 1983c], mucus hypersecretion [Coles, Neill and Reid 1984] and the increased release of mediators from mast cells [Frewtell et al 1982]. CGRP may also implicated in asthma, especially if it is co-released with substance P as it is a potent bronchoconstrictor and vasodilator [Lundberg et al 1983b, Lundberg et al 1985a].

Therefore, in addition to inflammatory and immunological mechanisms, nervous mechanisms are known to play an important role in bronchial hyper-reactivity and there are several ways in which a possible dysfunction in the NANC system could lead to the symptoms of asthma. The present study examines the distribution of nerve fibres, identified immunohistochemically with antibodies to the specific neural marker, PGP 9.5, in the epithelium of normal and asthmatic human lung, and also looks at the distribution of substance P and CGRP immunoreactivity.

1.3. THE INNERVATION OF NORMAL LUNG.

The gross nerve supply of the human lung was first described as long ago as 1663 by Thomas Bartholinus and has since been found, by the application of silver staining methods, to be similar in many species [Berkley 1893, Spencer and Leof 1964, Nagaishi 1972].

1.3.1. Afferent pathways.

Several different types of afferent nerve terminal have been described in the airways, their purpose being to send sensory information via the vagus nerve so that appropriate reflex change may be initiated in airway function e.g. breathing rate, smooth muscle tone etc. A large proportion of the fibres supplying the numerous sensory nerve endings in the lung travel in the vagus nerve and have their origin in the jugular-nodose ganglia [Widdicome 1977]. The afferent nerves in the upper respiratory tract have been reported to emanate from the trigeminal, jugular-nodose and cervical dorsal root ganglia [Uddman and Sundler 1987]. Spinal afferents supply the trachea and lower airways and have their origin in the thoracic dorsal root ganglia (T1-T6).

Physiological studies have indicated three main types of sensory nerves in the lung [Widdicome 1986]. Neuroendocrine cells have recently been ascribed a sensory role [Pack and Widdicome 1984, Lauwreyns et al 1985] and so are included here as a fourth group in the sensory system:

i. Slow adapting stretch receptors

Located in the intrapulmonary airways just beneath the airway epithelium

and in the airway smooth muscle. Excitation gives rise to the 'Hering-Breuer' reflex [Bradley 1977].

ii. Rapidly adapting 'irritant' stretch receptors

Those located in the extrapulmonary airway in the airway epithelium are thought to be 'cough' receptors [Mills et al 1970]. Irritant receptors are also located in intrapulmonary airway epithelium; stimulation of these receptors produces reflex bronchoconstriction [Widdicome 1981].

iii. 'C'-fibre endings

Located in deep lung and are possibly the 'J'-receptor found in alveoli or bronchioli [Coleridge and Coleridge 1984]. Stimulation produces bradycardia, pulmonary hypertension and rapid, shallow breathing.

iv. Neuroendocrine cells

In the lung these cells have been referred to by several names including amine-containing cells, as they were demonstrated to contain 5-hydroxy-tryptamine and catecholamines, notably dopamine [Eaton and Fedde 1977], endocrine cells [Hage 1980], and Kultschitzky-like cells [Bensch et al 1965]. It is accepted that all these names refer to the same cell type [Pack and Widdicome 1984] and the cells are thought to be part of the APUD (amine precursor uptake and decarboxylation) series described by Pearse [Pearse 1969]. Electron microscopic techniques have revealed the presence of dense-cored vesicles granules in the cells [Hage 1980], which, it has since been suggested, contain the amines observed at the light microscope level. Various peptides, including bombesin, CGRP, leu-enkephalin and calcitonin have also been described in this cell type [Cutz et al 1981]. Neuroendocrine cells are present in small clusters in the bronchioli of the foetus and neonate

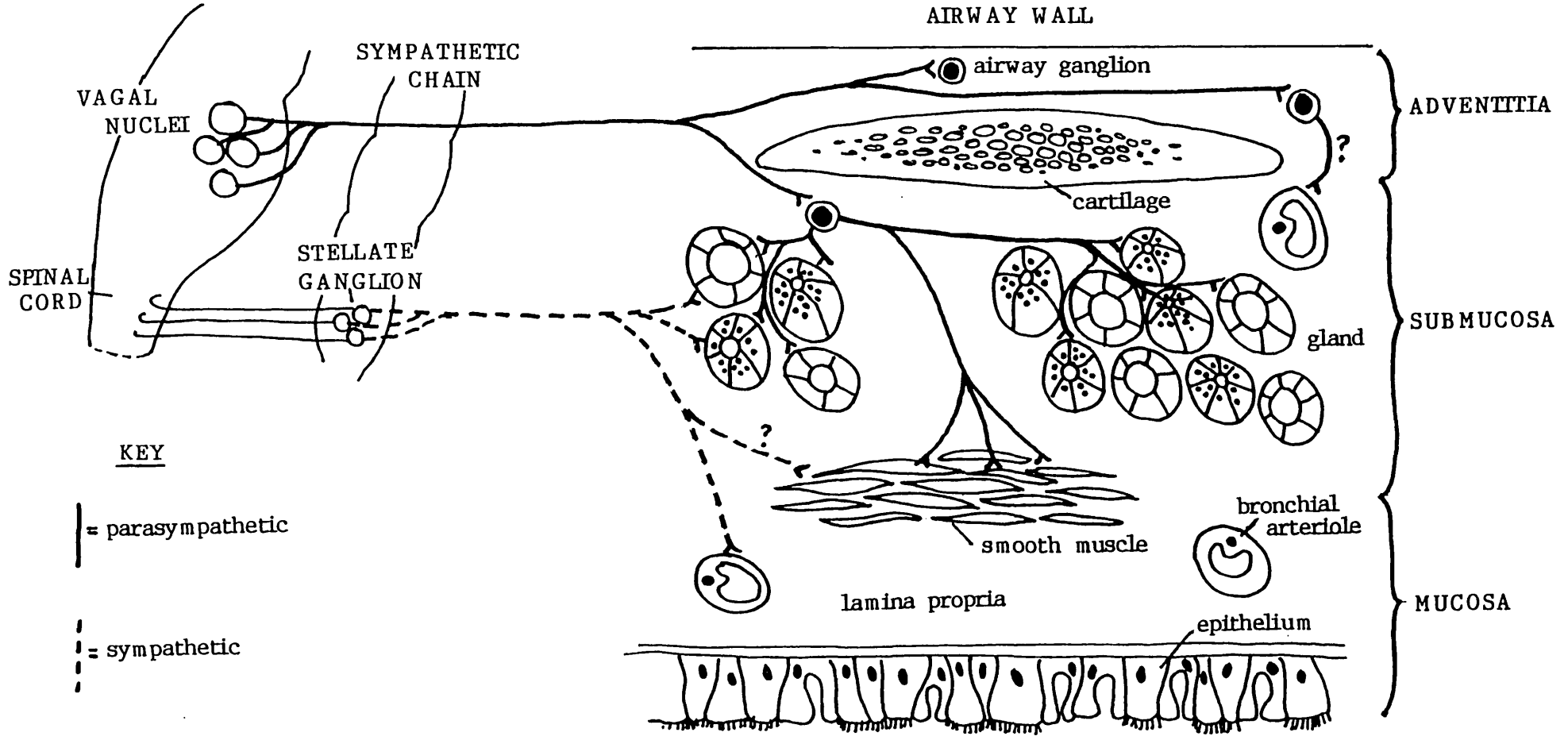
and are referred to as 'neuroepithelial bodies' [Lauweryns and Peuskens 1962]. They disappear as the infant matures and are quite rare in adult human epithelium, suggesting that they have a role in development [Hage et al 1977]. Neuroendocrine cells are found in close contact with afferent nerve terminals in animals [Jeffery and Reid 1973, Lauweryns et al 1985]

Innervation of the lung has been described in the foetus and neonate [Sheppard et al 1984a]. In the latter study, the appearance of a marker of nervous tissue, neuron specific enolase (NSE), in neuroblasts, nerve fibres and endocrine cells was demonstrated as early as 8 weeks gestation. Cholinergic nerves appear between 10 and 12 weeks gestation and dopamine β-hydroxylase (DβH), VIP and calcitonin appear later at 20 weeks. NPY has also been shown to appear at 20 weeks gestation [Sheppard et al 1984b]. Bombesin-immunoreactive endocrine cells have been demonstated at 8 weeks gestation [Sheppard et al 1984a], a finding consistent with the hypothetical role of these cells in pulmonary development [Wharton et al 1978].

1.3.2. Efferent pathways. (see fig.1.4)

Preganglionic parasympathetic fibres from the vagal nuclei descend in the vagus nerve, synapsing on small ganglionic formations along the vagus nerve and with ganglia located in the airway walls. Short postganglionic fibres arising from them innervate specific effector structures in the airway walls. In contrast, in the sympathetic system, short pre-ganglionic fibres leave the spinal cord and synapse with the paravertebral stellate ganglia (i.e. the fusion of the lower cervical and first thoracic ganglia) which give rise to long post-ganglionic sympathetic fibres which innervate similar effector structures. These nerve bundles enter the lung at the hilum; the large bundles of nerve fibres are located mainly in the posterior membranous

Fig.1.4 Schematic diagram of parasympathetic and sympathetic pathways to the airway wall



portion of the extrapulmonary airways [Fischer 1964]. On entering the lung the nerves divide to give rise to plexi lying internal and external to the cartilage plates. That external to cartilage consists of both pre- and post-ganglionic fibres, whilst the internal plexus consists mainly of post-ganglionic fibres. The majority of small parasympathetic ganglia are therefore located outside the cartilage. The nerve fibres show a general reduction in density as the airways divide. Nerve bundles are found as far as the bronchioles and early light microscopic reports of alveolar innervation [Hirsch et al 1968] have been confirmed by electron microscopic reports, but the innervation is much more sparse than previously thought [Fox et al 1980].

1.3.3. Innervation of effector structures in the airway wall.

1.3.3.1. Surface epithelium.

The early use of silver stains demonstrated a rich innervation of the bronchial epithelium in several species including man [Larsell and Dow 1933, Elftman 1943, Fischer 1964]. Most workers suggested a sensory role except Fischer, who suggested that they might represent a motor supply to epithelial goblet cells. Histochemical studies, however, have failed to demonstrate either cholinergic or adrenergic fibres in the epithelium suggesting that the fibres are more likely to be sensory in function [Fillenz and Woods 1970]. At the electron microscope level the human airway epithelium shows a sparsity of nerve profiles, in contrast to other species, although the lamina propria shows a dense supply of both myelinated and unmyelinated nerve fibres [Jeffery and Reid 1973, Das et al 1979, Jeffery 1982]. Intraepithelial nerves have been found in main and segmental bronchi [Laitenen 1985b]. It is possible that these profiles represent irritant receptors or 'C'-fibre endings

as they contain large numbers of mitochondria and neurofilaments which have been described as criteria for afferent nerve terminals [King et al 1974].

Substance P-immunoreactive nerve fibres have been located within human airway epithelium and in the mucosa, running close to the basement membrane [Lundberg et al 1984], although other workers claim that human lung is negative for substance P [Laitenen et al 1983, Laitenen 1985a]. CGRP has been demonstrated in rat neuroepithelial bodies and neuroendocrine cells in dense cored vesicles [Lauweryns and van Ranst 1987] and in nerve fibres in guinea-pig airway epithelium [Lundberg et al 1985a], although there are no reports of its presence in human airway epithelium. Although VIP receptors have been localised in human airway epithelium [Carstairs and Barnes 1986], there are no reports of corresponding VIP innervation. Similarly there are no reports of the presence of NPY in human airway epithelium.

1.3.3.2. Tracheobronchial submucosal glands.

Cholinergic innervation of mammalian submucosal glands has been demonstrated by acetylcholinesterase histochemistry, However, important species differences exist e.g. in sheep there are dense acetylcholinesterase-positive fibres around airway submucosal glands, whereas in calf and rabbit there are none [Mann 1971]. Larsell and Dow describe the gland nerve supply as being derived from adjacent intra-pulmonary ganglia [Larsell and Dow 1933]. Electron microscope studies show agranular vesicles containing nerve terminals adjacent to submucosal gland cells [Meyrick and Reid 1970] although in human glands intra-acinar penetration is rare compared to cat [Jeffery 1982]. Adrenergic innervation is also present close to bronchial submucosal glands [Sheppard et al 1983, Pack and Richardson 1984] but is

more sparse than the cholinergic supply [Mann 1971]. Spencer and Leof describe the adrenergic supply to the glands as being derived from the adrenergic nerves around the bronchial arteries [Spencer and Leof 1964].

VIP-immunoreactive fibres have been described in human airway submucosal gland [Uddman et al 1978, Laitenen 1985a]. NPY-immunoreactivity has been described in the tracheobronchial submucosal glands of the guinea-pig [Uddman et al 1984]. However, little is known about the distribution of this or other neuropeptides to human airway submucosal glands.

1.3.3.3. Bronchial smooth muscle.

The parasympathetic nervous system provides the major motor drive to the airways in all mammals including man and plays an important role in the regulation of smooth muscle tone [Nadel 1980]. Cholinergic nerves from the peribronchial plexus penetrate the bronchial smooth muscle terminating in a simple end knob (motor) or smooth muscle spindle (sensory) [Larsell 1922].

Acetylcholinesterase-positive fibres have been demonstrated in the bronchial smooth muscle of most mammals including man [Mann 1971, El-Bermani 1973, Richardson and Ferguson 1979]. Electron microscope studies complement these findings, demonstrating nerve profiles close to smooth muscle fibres: each ending contains small agranular vesicles, thought to contain acetylcholine [Richardson and Ferguson 1979]. Motor endings in human smooth muscle do not show synaptic specialisation: the neurotransmitter therefore reaches the muscle cells by diffusion. Connections of the nexus (gap junction) type are observed between the smooth muscle cells in human airway [Richardson and Ferguson 1979, Jeffery 1982] suggesting that they are electrically coupled. The release of neurotransmitter from one nerve fibre can therefore have widespread effects throughout the smooth

muscle.

Adrenergic innervation of human bronchial smooth muscle is very sparse, in contrast to most other mammals e.g. cat where it is mainly located in the upper trachea [Richardson and Beland 1976, El-Bermani 1978, Richardson and Ferguson 1979, Laitenen 1985a]. Immunocytochemistry using antibodies to dopamine β-hydroxylase, an enzyme involved in the metabolism of noradrenaline, confirm these findings [Sheppard et al 1983]. There is however still some confusion in the literature as to whether the smooth muscle, in airways smaller than the trachea and main bronchus, is innervated by adrenergic nerves [Partanen et al 1982, Sheppard et al 1983, Pack and Richardson 1984, Laitenen 1985a]. The present study makes an attempt to clarify this. The apparent sparsity of adrenergic innervation of the smooth muscle would seem to indicate that this is not the major controlling influence over smooth muscle relaxation. Non-adrenergic inhibitory innervation therefore seems to be the only direct neurodilator mechanism in the absence of sympathetic innervation. VIP-positive nerve fibres have been localised in human airway smooth muscle [Uddman et al 1978, Dey et al 1981]. Some motor endings containing large dense-cored vesicles, thought to be representative of peptidergic nerve profiles, have been found in mammalian smooth muscle [Jeffery 1982] and these vesicles have been reported to co-exist with the smaller agranular vesicles characteristic of cholinergic nerves [Dey et al 1981, Laitenen 1985a]. NPY-positive fibres have been described in the bronchial smooth muscle of guinea-pig [Uddman et al 1984] and man [Sheppard et al 1984b].

Substance P has also been demonstrated in small amounts in the airway smooth muscle of several species [Polak and Bloom 1982, Lundberg et al 1984]. However there is some debate about the existence of these fibres in

the airway smooth muscle of man [Laitenen et al 1983, Lundberg et al 1984, Laitenen 1985a].

1.3.3.4. Vasculature.

Acetylcholinesterase-positive nerve fibres have been demonstrated around the bronchial arteries of several species [Spencer and Leof 1964]. However, in man some workers report an absence and others only a sparse presence [Mann 1971, Partanen et al 1982, Laitenen 1985a]. In contrast, the numbers of adrenergic fibres greatly exceed the numbers of cholinergic fibres around the vessels [Mann 1971, Partanen et al 1982, Laitenen 1985a].

VIP has been localised around vessels in the airways [Uddman et al 1978, Laitenen 1985a]. In describing the distribution of NPY-immunoreactive fibres, Uddman and colleagues note that most are situated around the thick walled vessels of the guinea-pig respiratory mucosa [Uddman et al 1984]. In man NPY-immunoreactive fibres have been described around both tracheobronchial and pulmonary arteries and veins down to the level of capillaries [Sheppard et al 1984b].

Substance P has been reported around human airway vasculature [Lundberg 1984]. The relative densities of these types of nerve fibre in association with airway vasculature is unknown. The present study will examine in detail the relative distribution of adrenergic, cholinergic and peptidergic components supplying both bronchial (i.e. intrachondrial) and pulmonary (i.e. extrachondrial) arterioles/capillaries.

1.3.3.5. Airway ganglia.

Silver impregnation studies have demonstrated ganglia in the airway wall: the number declines with increasing airway generation until the level of the small bronchi, beyond which only nerve bundles occur [Spencer and Leof 1964, Fischer 1964]. More recent studies utilising antibodies to NSE show that the ganglia consist of approximately 20 or less nerve cells and occasional isolated neural cells may occur [Sheppard et al 1983]. The ganglia are assumed to be part of the parasympathetic nerve supply and in support of this they have been shown to exhibit acetylcholinesterase positivity [Mann 1971, Sheppard et al 1983, Laitenen 1985a]. The ganglia give rise to post-ganglionic cholinergic fibres which supply the airway mucosa. Clusters of cholinergic ganglia exhibiting catecholamine activity have been reported [Jacobwitz 1978, Knight 1980] and in the cat, parasympathetic ganglia exhibiting immunocytochemical reactivity for VIP [Uddman et al 1980] have been reported. In man, airway ganglia show a positive immunoreaction for substance P [Lundberg et al 1984].

Electron microscope studies show that the nerve ganglia are surrounded by a capsule which also contains satellite cells, bundles of myelinated and unmyelinated nerve fibres and the occasional blood vessel [Jeffery 1982]. Granulated cells resembling the Kulschitsky/Feyrter cell have been found [Richardson and Ferguson 1979, Knight 1980, Jeffery 1982]. Such cells contain large numbers of dense-cored vesicles which tend to have a peripheral location [Uddman 1978]. Such vesicles could contain the catecholamines and peptides observed at the light microscope level.

The distribution of the different nerve types of the autonomic nervous system has been determined histochemically in several mammals and major

species differences in the extent and type of innervation have been identified [Mann 1971]. The general pattern of innervation in human airways was established long ago [Larsell and Dow 1933, Gaylor 1934]. However, the innervation of specific effector organs is less well understood, especially in regard to the distribution of the NANC system. There is still controversy about the exact distribution of adrenergic and cholinergic innervation to various effector structures in the airway wall i.e. airway smooth muscle and vasculature respectively. As yet, there are few studies on the NANC innervation of human airways, and of those that have been published few are comprehensive. Again there is controversy over the distribution of peptides in human lung, for example, whether or not substance P exists in any appreciable quantity in the airways of man [Lundberg et al 1984, Laitenen 1985a]

The present work aims to provide a detailed study of both the classical sympathetic and parasympathetic supply to the airways and the distribution of the neuropeptides, substance P, CGRP, VIP and NPY.

1.4. TECHNIQUES TO DELINEATE THE NERVOUS SYSTEM.

The oldest methods used to delineate the gross distribution of the nerve supply to various organs employ silver stains. In these methods, silver salts are precipitated onto the nerve fibres and a photographic developer solution or other reducing agent is used to stain the nerves black. Introduced originally by Golgi in the late 1800's, these methods are still widely used today. However, these methods are notoriously capricious: the stain is not specific and may also stain blood vessels, muscle nuclei and connective tissue elements such as reticulin and elastin. Alternative methods to stain nerves have been used e.g. Methylene blue, first described by Ehrlich in 1885. Unfortunately, the same of lack of specificity applies. More recent techniques utilise the application of antibodies to specific nerve components e.g. protein gene product 9.5 (PGP 9.5) and neuron specific enolase, and hence provide a consistent and reliable way of identifying nervous tissue. However, these techniques are of limited use in studying the anatomic relationship of specific nerve types to target cells. Techniques have been developed which stain either the neurotransmitter itself or the enzymes associated with its synthesis/breakdown: the last is used if the neurotransmitter is difficult to retain in the tissue. The three components of the autonomic nervous system (ANS) namely the sympathetic, parasympathetic and NANC systems may be demonstrated in histological sections using the following techniques:

1.4.1. The parasympathetic system; cholinesterase histochemistry.

The first method for the histochemical demonstration of cholinesterases was introduced by Gomori in 1948. Higher fatty acid esters of choline were used

as the substrate for the enzymes. The acid released in the hydrolysis of the ester forms a salt with cobalt present in the medium. This salt is converted to black cobalt sulphide by subsequent application of ammonium sulphide solution. The substrates used in this method, however, are not particularly specific for cholinesterases. In 1949 Koelle and Friedenwald showed that thiocholine esters are more specific substrates and subsequent methods of demonstrating cholinesterases histochemically are based on this. The reaction medium contains acetylthiocholine or butyrylthiocholine as the substrate, copper (as copper sulphate) as the 'capture agent' and glycine or citrate to chelate the copper and so minimise its inhibitory effect on the enzyme. The reaction is thought to proceed as follows (Bergner and Bayliss 1952):-

$$2\ CH_3COSCH_2CH_2N(CH_3)_3OH + (NH_2CH_2COO)_2Cu + H_2O \xrightarrow{\text{cholinesterase}}$$

acetylthiocholine — copper glycinate

$$Cu[SCH_2CH_2N(CH_3)_3OH]_2 + 2\ NH_2CH_2COOH + 2\ CH_3COOH$$

copper thiocholine — glycine — acetic acid

The copper thiocholine produced is visualised by 'development' in a sulphide containing solution to produce dark brown copper sulphide [Malgrem and Sylven 1955] or, as in the 'direct colouring' method of Karnovsky and Roots, by the precipitation of insoluble red-brown copper ferrocyanide, by the inclusion of potassium ferricyanide in the incubation medium [Karnovsky and Roots 1964]. A direct reaction between the copper and the ferricyanide is prevented by the inclusion of citrate which chelates the copper ions. The maximum pH for the reaction is 5.65 [Lewis 1961]. The advantage of the direct colouring method is that the reaction can be monitored throughout and the optimum reaction time easily established. The inclusion of specific substrates in the reaction medium is not in itself sufficient to distinguish

between acetyl- and butyrylcholinesterase; butyrylcholinesterase will hydrolyse both acetylthiocholine and butyrylthiocholine. By the inclusion of appropriate inhibitors, acetylcholinesterase and 'pseudocholinesterase' (butyrylcholinesterase) may be distinguished [Koelle 1950, 1951]. Various inhibitors have been used to reduce the activity of the unwanted enzymes. In the original method Karnovsky and Roots used eserine sulphate (physostigmine) which distinguishes cholinesterases from other esterases at a concentration of 10^{-5}M [Easson and Stedman 1937, Mendel et al 1943]. Other inhibitors have been used to distinguish between different types of cholinesterase. One of the earliest was 'DFP' (di-isopropylphosphoro-fluoridate), and in most species butyrylcholinesterase is at least ten times more sensitive than acetylcholinesterase to inhibition by DFP [Ecobichon and Comeau 1973, Mendel and Myers 1955]. However, there is an overlap in the sensitivities, and the concentration needed to completely inhibit butyrylcholinesterase may also cause inhibition of acetylcholinesterase activity. DFP is also extemely toxic, it is an irreversible inhibitor, volatile and may be absorbed by the skin. Its use should therefore be avoided. Butyrylcholinesterase may be selectively inhibited by a number of different compounds such as iso-OMPA (tetraisopropylpyrophosphoramide) and ethopropazine hydrochloride [Silver 1974]. Acetylcholinesterase may be selectivly inhibited by BW284C51 [1:5-bis (4-allyldimethylammonium-phenyl)-pentan-3-one-dibromide].

These methods rely on the use of fresh or fresh frozen tissue where the structural integrity of the cholinesterase enzyme is maintained. Fixation by standard cross-linking reagents, such as formalin, will reduce enzyme activity. However, to demonstrate the enzyme distribution in relation to the structure of the tissue, the morphology of the section must be maintained. Cholinesterases are relatively resistant to fixation, provided it is done in the

cold (4°C) and for a short period of time (3-5 hr max.) (Taxi 1952, Fukuda and Koelle 1959, Austin and Phillis 1965), therefore a balance between the preservation of enzyme activity and tissue morphology needs to be acheived.

1.4.2. The sympathetic system: Induced fluorescence of catecholamines.

Catecholamines (noradrenaline, adrenaline, DOPA, and 5-Hydroxytryptamine) were originally localised in tissue sections by the application of the formaldehyde-induced fluorescence technique (FIF) [Falck and Owman 1965], which produces a blue-green fluorophore, 3,4 dihydroisoquinoline, from noradrenaline and/or dopamine. The excitation maximum for this fluorophore is 415nm and the emission maximum is at 480nm. However, this method requires exacting conditions of humidity and temperature. Simpler methods of visualising catecholamines in biological tissues were therefore sought. Reaction of noradrenaline with aqueous glyoxylic acid under vigourous conditions was found to produce the same fluorophore as that produced in the FIF technique [Axelsson, Bjorklund and Lindvall 1972]. Subsequently, simpler methods were introduced such as the 'SPG' method of De la Torre and Surgeon [1976] where glyoxylic acid is dissolved in an aqueous sucrose-phosphate buffer; the sections are immersed in the solution and then rapidly air-dried before heating at high temperature on a hotplate (80°C) to produce the fluorophore. The fluorophore can be removed with water [Furness and Costa 1975]. These methods rely on the rapid and adequate preservation of the neurotransmitter within the tissue. Therefore it is important that the samples are frozen as quickly as possible after removal from the body. Various methods to improve the localisation or intensity of reaction of the catecholamines have been suggested; these include incubation of the tissue

with -methylnoradrenaline [O'Donnell et al 1978] and amine precursors such as L-DOPA [Pack and Richardson 1984].

1.4.3. The NANC system; immunohistochemistry.

Immunocytochemistry is the identification of a tissue constituent in situ by means of a specific antibody tagged by a microscopically visible label. Because an antibody - antigen reaction is specific, highly selective identification of individual cell and tissue constituents is made possible.

Immunohistochemistry originated with Coons who was the first to label an antibody with a fluorescent, green dye and use it to identify an antigen in tissue sections [Coons et al 1941, Coons and Kaplan 1950]. The label used was fluoroscein isocyanate, this was soon replaced by fluoroscein isothiocyanate (FITC), which was found to be more easily conjugated to the antibody molecule. The label is observed microscopically at an excitation wavelength of 490nm using a 515nm suppresion filter to cut down non-specific autofluorescence. A variety of different labels are now used e.g. the red fluorescent dye rhodamine isothiocyanate and enzymes such as peroxidase [Nakane and Pierce 1966]. Antibodies have also been labelled with radioactive elements, the consequent immunoreaction being visualised by autoradiography. Many antibodies are now commercially available, including some that act as general nerve markers by labelling nerve components such as neuron specific enolase (an isoenzyme of the glycolytic enzyme enolase present in both central and peripheral neurones and endocrine cells [Marangos and Schmechel 1980, Schmechel et al 1979] and protein gene-product 9.5 (a 24kD protein present in neurons in the brain) [Thompson et al 1983]. Markers are also available which label specific neuropeptides e.g. VIP or enzymes involved with the synthesis/breakdown of neurotransmitters such as

tyrosine hydroxylase. Successful immunostaining is dependent not only on antigen preservation but also preservation of tissue morphology, to allow localisation of substance of interest. The preservation of neuropeptide antigenicity is achieved using fixatives such as p-benzoquinone, which promote weak cross-linking or by rapid freezing of fresh tissue, followed by post-fixation if necessary. High temperatures and prolonged fixation in strong cross-linking reagents may reduce staining potential by damaging the peptide tertiary structure or by reducing its availability to the antibody. Paraffin embedding destroys antigenicity to some extent. Improved antibodies and the application of more sensitive staining techniques have helped to reduce this problem.

1.4.3.1. Indirect immunofluorescence.(see fig.1.5)

The method of direct labelling of tissue antigens was modified by Coons in 1955 [Coons et al 1955]; unlabelled primary antibodies were incubated with the tissue sections which were then visualised by the application of an FITC-conjugated second layer antibody. The second layer antibody is raised against the Fc portion of the immunoglobulins of the donor species in which the primary antibody was raised. The advantage of this technique is that at least two second layer antibodies will bind to each primary antibody molecule resulting in amplification of the primary reaction. However, there may also be non-specific attachment of second layer antibodies by hydrophobic and electrostatic bonding to other tissue components: the unwanted reaction can be blocked by first incubating the sections with non-immune serum from the same species in which the second layer antibodies were raised [Polak and Van Noorden 1983].

Fig.1.5 Indirect Immunofluorescence

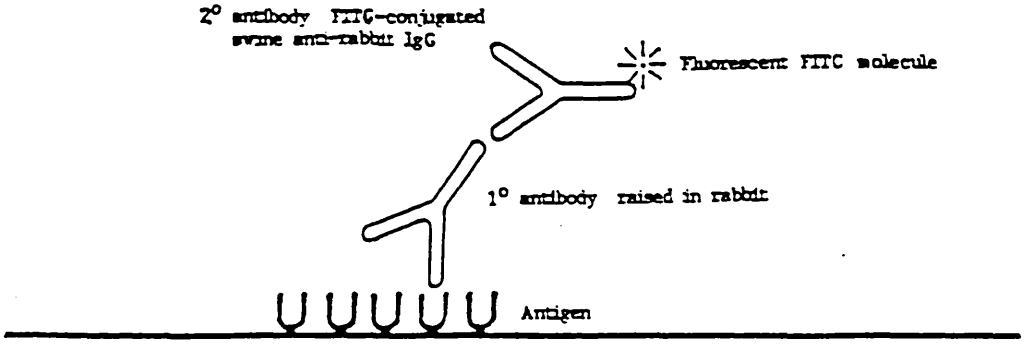


Fig.1.6 Avidin-Biotin Enhancement

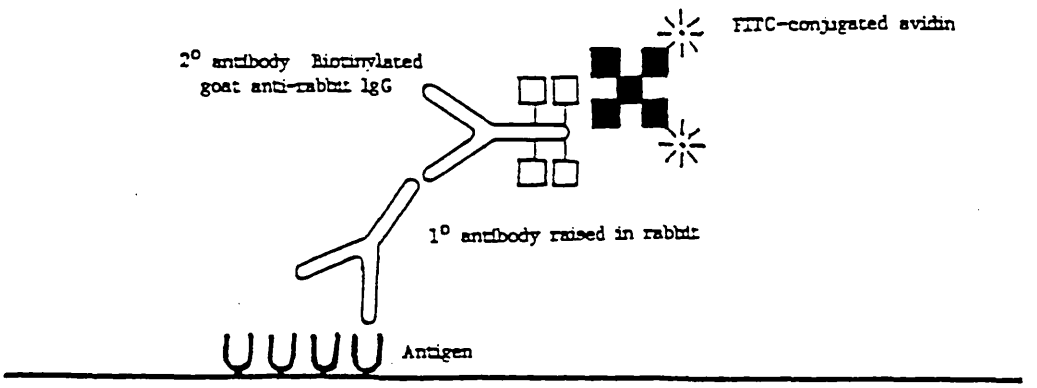


Fig.1.7 Peroxidase - Anti-Peroxidase

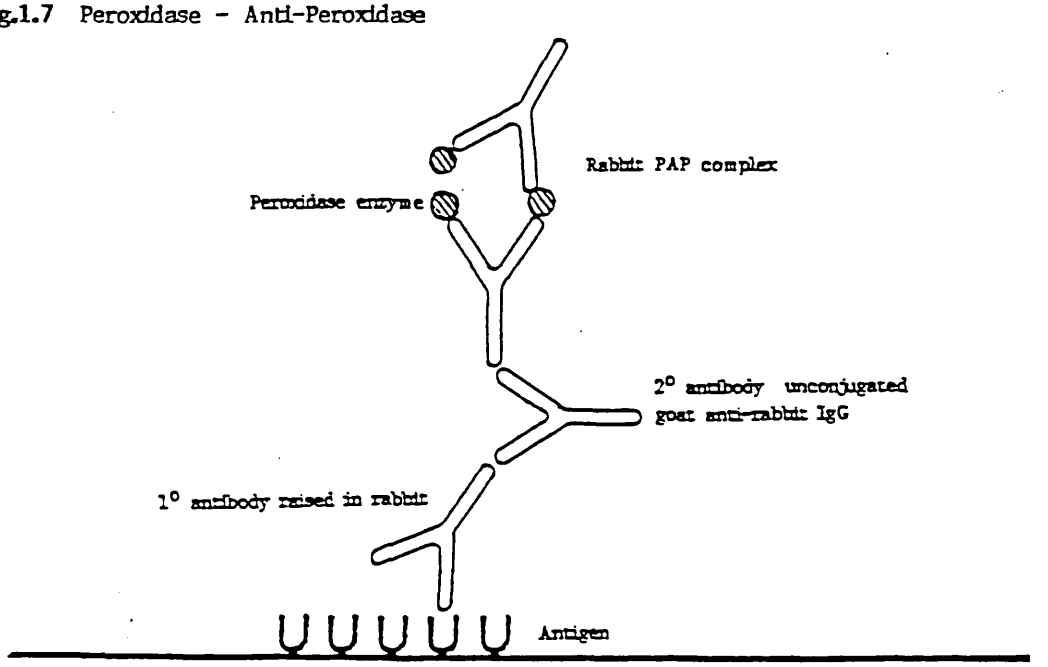
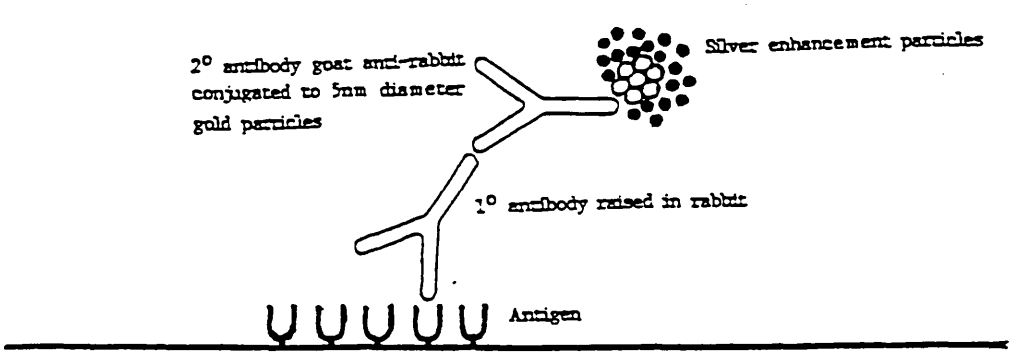


Fig.1.8 Immunogold/Silver Enhancement



1.4.3.2. Avidin-Biotin enhancement. (see fig.1.6)

With the increasing field of application of antibody staining techniques, and the need to detect weaker antigens in fixed tissue sections, more sensitive methods of antigen detection were developed. One such technique utilises the naturally occuring high binding affinity ($Ka=10^{-5}$ M^{-1}) between molecules of avidin and biotin. Avidin is a high molecular weight (67 Kdal) glycoprotein contained in egg white. Biotin (vitamin H) is of low molecular weight (244 dal) and found in egg yolk. Each molecule of avidin has four binding sites for biotin. The extraordinary binding affinity of avidin for biotin was originally harnessed in biochemical extraction procedures. One of the first applications in immunocytochemistry was made by Heggeness and Ash, who in 1977 utilised fluoroscein-conjugated avidin and biotin labelled rabbit anti-human myosin IgG to label myosin components in non-muscle cells. To prevent stearic hinderance of binding between avidin and antibody conjugated biotin, a 'spacer arm' is added to the biotin antibody complex. This consists of a small molecule linking the amino end of the biotin to the carboxyl end of the antibody, widening the distance between the two. Maximum sensitivity of reaction is thus achieved. Unwanted reactions may occur between avidin and endogenous biotin in the tissue but this may be prevented by prior incubation of the tissue with free avidin to block the endogenous biotin in the tissue and then with free biotin to bind the excess avidin [Wood and Warnke 1981]

1.4.3.3. Peroxidase anti-peroxidase. (see fig.1.7)

An alternative way of providing amplification of the primary immunoreaction came with the development of the indirect peroxidase

technique. The original use of peroxidase in immunocytochemistry was as an enzyme label for the second layer antibody in the indirect method [Nakane and Pierce 1966]; the basic method was modified in 1979 by Sternberger. The primary antibody (usually raised in rabbit) is applied to the tissue followed by unconjugated goat anti-rabbit IgG, added in excess. To this the 'PAP' (peroxidase, anti-peroxidase) complex is added. The complex is the enzyme peroxidase together with an antibody to the peroxidase. An excess of second layer antibody ensures there are plenty of free binding sites on the unoccupied halves of the 'Fab' portion of the second layer antibody molecule for the rabbit PAP complex, thus providing a system of amplification of the reaction between the primary antibody and tissue antigen. The peroxidase complex is visualised by development with the diaminobenzidine (DAB)-hydrogen peroxidase reaction of Graham and Karnovsky [1966]. The reaction product is an insoluble dark brown precipitate and the sections may be counterstained with routine histological stains. The reaction is more sensitive than the indirect method because the peroxidase is not chemically bound to the immunoglobulin of the second layer (which reduces the activity of the enzyme) but immunologically bound which does not affect the peroxidase activity. However, it should be noted that the enzyme reaction is inhibited by sodium azide, a commonly used anti-fungal agent in commercially available antiserum: care should therefore be taken to ensure all reagents are sodium azide free.

The reaction is highly sensitive, allowing high dilution of the primary antibody and thereby reducing the possibility of non-specific reaction. However, there may be a reaction with endogenous peroxidase which should therefore be blocked by preincubation of the section with hydrogen peroxidase [Polak and Van Noorden 1983]. Also DAB is thought to be carcinogenic and although there are alternatives (3 amino-9-ethylcarbazole

or 4 chlorol-1-napthol, they are alcohol soluble and can therefore only be counterstained and mounted with aqueous chemicals.

1.4.3.4. Immunogold/Silver enhancement (see fig.1.8).

Colloidal gold was originally used as a label at the electron microscopic level [Faulk and Taylor 1971]. However, for use in light microscopy, high concentrations of primary antibody are required to attract sufficient gold-labelled secondary antibody to visualise the reaction [Geoghegan et al 1978, Gu et al 1981]. The method was adapted by Holgate in 1983, who combined Geoghegan's method and another method originated by Danscher [1981] for the detection of gold in biological tissues. The result was an improved immunogold technique, whereby the distribution of gold-labelled antibody is visualised by the precipitation of silver around the gold particles from silver lactate solution. The precipitate is fixed with conventional photographic fixer solution. Holgate claims the method gives greater sensitivity than PAP methods [Holgate et al 1983]. More recently the method has been applied to the detection of small concentrations of neuropeptides present in routinely fixed, paraffin-embedded tissues [Springall et al 1984]. Since the original method of immunogold/silver staining was published, commercial staining kits have become available. They employ light insensitive silver enhancement solutions, which simplify the method for routine use.

1.4.4. Electron microscopic techniques.

Immunogold labelled antibodies may also be employed at the electron microscope level for specific labelling, otherwise nerve types are usually classified according to the type of granule they contain. This method of

classification, however, is controversial [see Gibbins 1982]. Cholinergic nerves are thought to contain small (30-60nm) agranular vesicles and adrenergic nerve profiles, small (30-60nm) granular vescicles. Peptidergic nerves are thought to contain large (80-100nm) dense cored vesicles, whereas purinergic nerves contain large (80-200nm) opaque vesicles.

1.4.5. Radioimmunoassay (RIA) techniques.

The first RIA was developed by Yalow and Berson [see Yalow and Berson 1959]. RIA depends upon a quantitative reaction between an antigen and its antibody. A known amount of antigen labelled with radioactive iodine competes with an unknown amount of unlabelled antigen for the specific antibody of interest in the sample to be investigated. The amount of antigen or peptide in the sample is measured by the ratio of unbound antigen to I_2-labelled antigen bound to antibody. Modern semi-automated techniques are much quicker and easier to use than the original bioassay methods and the accuracy of the techniques is constantly being improved. Kits are now widely available with antisera of high avidity against specified regions of the antigen in question.

1.4.6. Spectrophotometric determination of cholinesterases.

The basic techniques for measuring cholinesterase activity have long been used. These include gasometric methods which measure the evolution of CO_2 from bicarbonate Ringer's solution in a Warburg manometer, titimetric methods where the acid released by hydrolysis is titrated against alkali [Jacobsen et al 1957], measurement of the change in pH which occurs as the acid is liberated, either measured directly in electrometric recording or

indirectly by photometric recording of the colour change of an indicator, such as phenol red [Michel 1949]. Colorimetric methods have also been used where the reaction product undergoes a further reaction to produce a coloured end-product, the appearance of which is monitored by a spectrophotometer [Ellman et al 1961]. The last mentioned technique has been widely used as it is simple, accurate and flexible, and can be used to measure either acetyl- or butyrylcholinesterase. In the original method, either acetylthiocholine or butyrylthiocholine is used as the substrate. Thiocholine is released on hydrolysis by the cholinesterase enzyme and then reacts with 5:5-dithiobis-2-nitrobenzoate (DTNB) to give the yellow anion of 5-thio-2-nitrobenzoic acid. Each mole of anion produced represents hydrolysis of 1 mole of substrate. The rate of production of the coloured anion is recorded at 412nm on a spectrophotometer and various corrections must be made to allow for the absorbance of the enzyme suspension and the spontaneous hydrolysis of the substrate. Ellman and colleagues suggest that the rate of hydrolysis given by this method can be taken as a good indication of the rate of hydrolysis of acetylcholine.

1.5. AIM AND OBJECTIVES OF THE PRESENT STUDY.

Aim:-

To delineate the autonomic innervation of normal human lung, including the parasympathetic, sympathetic and peptidergic supply to the major effector structures in the airway wall and to describe any changes that may occur in disease.

Specific Objectives:-

1.i. To analyse the types of nerve and their numbers in the human airway lining epithelium.

ii. To study the peptidergic component of the human submucosal gland.

iii. To determine if there is a sympathetic supply to human bronchial smooth muscle.

iv. To study the distribution of butyrylcholinesterase in the airways.

v. To study the innervation of bronchial (i.e. intrachondrial) and pulmonary (i.e. extrachondrial) arteries/arterioles/capillaries.

vi. To examine the parasympathetic airway ganglia for an adrenergic or peptidergic component.

2. To identify any differences in innervation that may occur at different airway level generations in the 'normal' human lung.

3. To identify any changes in innervation that may occur in asthma and cystic fibrosis.

4. To quantify and validate any qualitative changes in these diseases using the following measurements:-

i. Quantitative and semi-quantitative assessment of the density of nerves

ii. Radioimmunoassay of peptides.

iii. Biochemical measurements of serum cholinesterase.

1.6. SUMMARY OF INTRODUCTION.

1. Several aspects of airway function are under autonomic control, including airway smooth muscle tone, submucosal gland secretion, epithelial function and bronchial vascular tone and permeability.

2. Neural control of human airways is more complex than has previously been recognised. In addition to the 'classical' adrenergic and cholinergic mechanisms, recent advances have identified a third component of the autonomic nervous system termed the 'non-adrenergic non-cholinergic' system. There are a number of potential neurotransmitters in this system including purines such as adenosine and ATP and several neuropeptides.

3. In the lung several peptides have been shown to be functionally active. These include vasoactive intestinal peptide, substance P, calcitonin gene-related peptide and neuropeptide Y.

4. More recent methods allow the elucidation of components of the autonomic nervous system:-

i. Cholinesterase histochemistry for the parasympathetic system.
ii. Glyoxylic acid-induced fluorescence of catecholamines for the sympathetic system.
iii. Immunohistochemistry of peptides for the non-adrenergic, non-cholinergic system.

5. The association of components of the autonomic nervous system with effector structures has been described in several mammalian species including man. There exists however some areas of uncertainty and

controversy:-

i. What is the exact distribution of nerves to the airway epithelium; are substance P and calcitonin gene-related peptide present in nerve fibres supplying effector structures in the airways of man?

ii. Is there a sympathetic component in the neural supply to human bronchial smooth muscle?

iii. What is the distribution and role of butyrylcholinesterase in the airways?

iv. What is the exact distribution of functionally active peptides in the lung?

v. What is the arrangement and type of nerves associated with airway vasculature?

vi. Are there additional components in the airway parasympathetic ganglia i.e. do other nerve types synapse with these ganglia?

6. Autonomic abnormality has been implicated in several disorders of man including cystic fibrosis and asthma in the lung.

7. Relatively few studies have been done on these conditions, mainly due to the lack of available tissue. Therefore, the role of the autonomic nervous system and especially the non-adrenergic, non-cholinergic component in the pathophysiology of these diseases remains an area of speculation.

8. The present study aims to clarify areas of doubt concerning the innervation of 'normal' human lung and to examine the distribution of the autonomic nervous system with particular reference to the peptidergic compononent in asthma and cystic fibrosis.

CHAPTER 2

MATERIAL AND METHODS

This chapter describes the materials and methods used: i) the patients and the way samples were obtained, ii) the methods of fixation and staining, iii) measurement of the peptide content in the samples by radioimmunoassay, iv) determination of butyrylcholinesterase activity in serum and v) the methods used to quantify the results and the statistical analyses used.

Details of measurements made on individual samples are given in chapter 3, Results.

The reagents used were supplied by BDH Ltd. unless otherwise stated. The addresses of the companies which supplied the reagents are given in Appendix 2.

2.1. PATIENT DATA.

Full details of smoking status, therapy and other relevant details of all the patients used in the study are shown in appendix 1. and are summarised as follows:-

'Normal' control tissue was obtained from elderly patients with an average age of 58 years (ranging from 27 - 78 years) undergoing resection for carcinoma (n=32), most were cigarette smokers (see appendix 1). To allow for changes occuring due to the age and smoking status of the 'normal' patients, tissue was also obtained from two alternative control groups, i) **'Young'**; patients in this group had an average age of 28 years (ranging from 3 - 62 years) and included patients with bronchitis, congenital emphysema and lung cysts (see appendix 1.); tissue was obtained at resection for these conditions (n=8), and ii) **'Bronchiectatic'**; tissue was taken from patients

(n=7) undergoing resection for bronchiectasis, none of whom smoked with an average age of 36 years (ranging from 9 - 67 years). Tissue was also obtained from patients with **cystic fibrosis** undergoing resection (n=1), transplant surgery (n=7) or at post-mortem (n=4) with an average age of 23 years (ranging from 3 - 29 years) and from patients with **asthma** at resection (n=1) and post-mortem (n=8), with an average age of 40 years (ranging from 13 - 64 years).

2.2. SOURCE OF TISSUE AND AIRWAY LEVELS.

2.2.1. Fresh human lung.

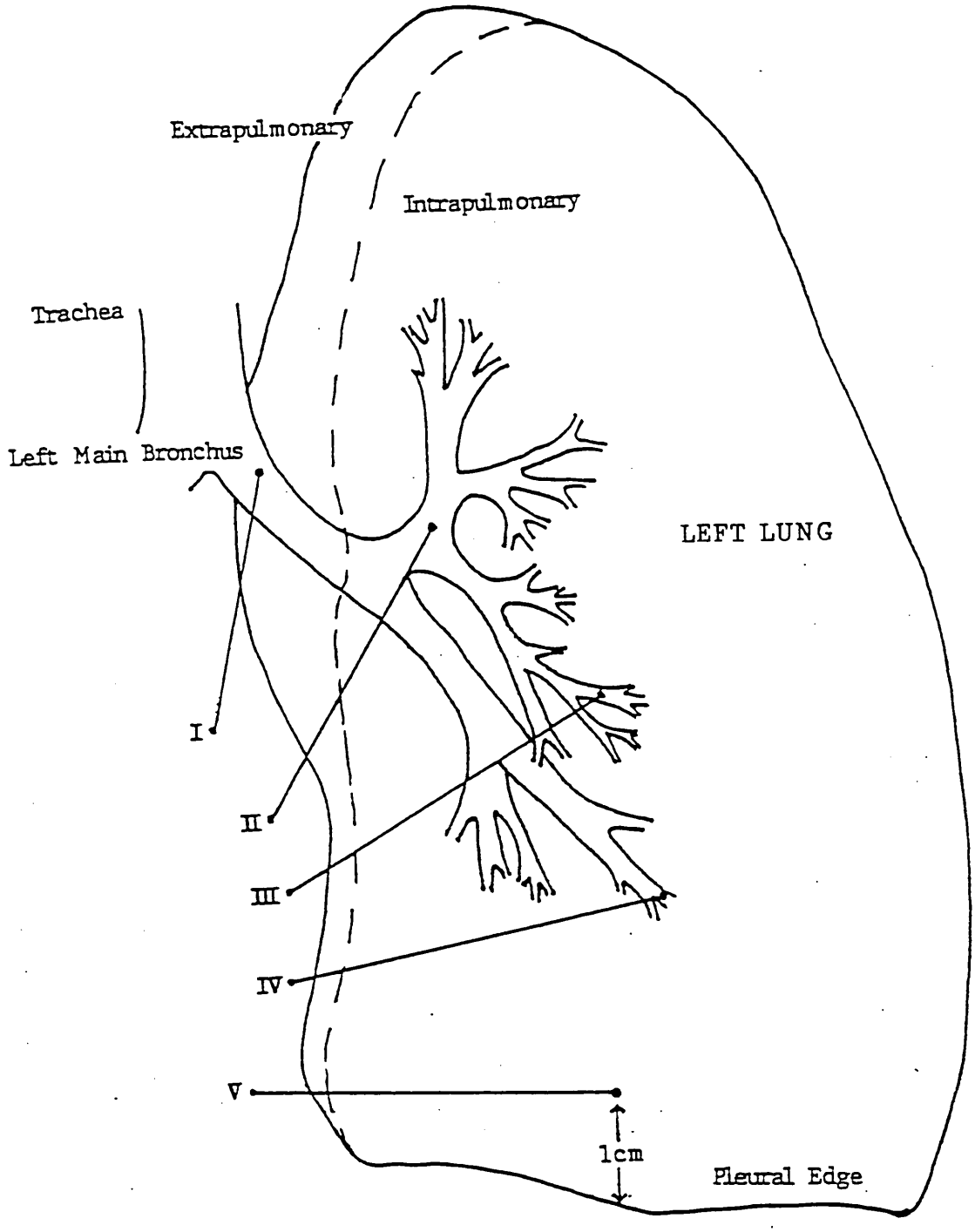
2.2.1.1.. Surgically resected tissue.

Tissue resected for carcinoma was collected from theatre usually less than half an hour after the lung had been removed from the patient (see appendix 1 for full details). The tissue obtained was examined by a pathologist before being dissected and fixed in the appropriate manner. Samples of the tissue obtained in this way were taken from 5 airway levels: Main bronchus (I), lobar bronchus (II), segmental airways (III), subsegmental airways (IV), and subpleural (V) (see fig.2.1).

2.2.1.2. Transplanted lung tissue.

Lung tissue from patients with cystic fibrosis who were undergoing transplant surgery was processed on site within half an hour of resection of the affected lung. Five airway levels (as before) were taken and frozen or fixed in the appropriate manner.

Fig.2.1 Diagram of Airway Levels



2.2.1.3. Post-mortem tissue

Tissue was obtained post mortem from cystic fibrosis patients usually within four hours of death (see appendix 1.)
Five airway levels (as before) were taken, the tissue being processed on site.

2.2.2. Archival material (pre-embedded tissue).

The tissue was obtained in the following form:-

i. Blocks of formalin-fixed, paraffin-embedded material, documented with a pathological report. Samples were obtained at resection from patients with carcinoma, (i.e. normal tissue from resection line) and bronchiectasis.

ii. Sections (6µm) of formalin-fixed, paraffin-embedded asthmatic tissue. These samples were taken from tissue obtained at early post-mortem (i.e. within 48hrs of death).

It was not possible to catagorise individual airway level generations in archival material: the samples were therefore assessed on the basis of whether the airway was intra- or extra pulmonary.

2.2.3. Control rat/guinea-pig tissue.

Tissue was obtained from animals killed by injection of 1-2mls 'hypnorm' (Janssen). Control tissues were taken from the carcases less than 10 minutes after death of the animal.

2.3. TISSUE PROCESSING.

The tissue was divided and each piece processed in one of the following ways:-

2.3.1. 'Snap' freezing.

Tissue was rinsed in Weymouth's culture medium (Gibco), blotted dry and immediately frozen in either liquid arcton cooled over liquid nitrogen (BOC) or in 'cryojet' (dichlorodifluoromethane, BDH). Frozen samples were placed in sterile plastic pots and stored in liquid nitrogen (½-1hr) until removed to the freezer cabinet for longer term storage: -70°C long term or -30°C short term (i.e. less than 1 month).

2.3.2. Benzoquinone-fixed tissue.

Tissue was rinsed in Weymouth's culture medium and placed in 0.4% benzoquinone in phosphate buffered saline (PBS, 0.01M, pH 7.4,) at 4°C for 1½-2hrs [Elde et al 1976, Bishop et al 1978]. Tissue fixed in this way was repeatedly rinsed in PBS (0.01M, pH 7.4) containing 15% sucrose for 1-2 days or until the buffer rinse no longer discoloured. The tissue was then snap frozen as above.

2.3.3. Formalin-fixed tissue.

Tissue was rinsed in Weymouth's culture medium and fixed in 10% neutral buffered formalin for 1-2 days. The tissue was then processed by dehydrating in 100% isopropanol and clearing in CNP 30 before embedding in

paraffin wax ('fibrowax', Raymond Lamb). Temperatures were kept below 60°C to preserve the neuropeptides in the tissue [Polak and van Noorden 1983].

2.3.4. Archival material.

Tissue was either fixed in 10% formal saline for up to 48hrs before being dehydrated in graded (70, 90 and 100%) ethanols, cleared in CNP 30 and embedded in paraffin wax ('fibrowax'), or fixed in 10% neutral buffered formalin for up to 48 hours before being dehydrated in 95% aqueous methanol (Spencer's Paints plc.), 100% ethanol (J. Burrough FAD Ltd.) and cleared in 100% chloroform (Infrakem Ltd.) before embedding in paraffin wax ('polywax', Difco Labs). The tissue was routinely processed in a 'Histokinette' (Reichert-Jung U.K. Ltd).

2.4. STAINING TECHNIQUES.

The following staining techniques were applied to the tissue samples.

2.4.1. Cholinesterase histochemistry.

Cholinesterase histochemistry was applied to human lung tissue to identify and locate cholinergic nerves and to examine the distribution of butyrylcholinesterase.

Tissue was stained for cholinesterases according to the method of El-Badawi and Schenk (1967) with modifications:-
18μm thick sections of fresh frozen tissue were cut on a cryostat (Brights) at -30°C, air-dried at room temperature for 5 minutes and post-fixed in 10% neutral buffered formalin at 4°C for 20-25 minutes. Following fixation, the sections were rinsed in distilled water and pre-incubated with the appropriate inhibitor in 0.2M sodium acetate-acetic acid buffer (pH 5.65) at room temperature for 30 minutes. After pre-incubation the slides were transferred directly to the incubation medium containing the appropriate inhibitor and substrate (see 'controls'), and left in an incubator for three hours at 37°C.

Incubation medium.

	substrate	25.0mg
60mM	sodium acetate	31.6ml
100mM	acetic acid	1.0ml
100mM	sodium citrate	2.4ml
30mM	cupric sulphate	5.0ml
	inhibitor	1.0ml
5mM	potassium ferricyanide	5.0ml
	distilled water	4.0ml
		50.0ml

The sections were then rinsed in distilled water and counterstained with Harris's haematoxylin, dehydrated in graded alcohols, cleared in xylene and mounted in DPX (see appendix 3). Alternate sections were stained with Alcian blue, pH 2.5/periodic acid Schiff to distinguish between acidic and neutral glycoproteins and aid in the identification of serous and mucous acini in the submucosal gland (see appendix 3).

Controls.

For acetylcholinesterase specificity (to delineate cholinergic nerves), 1.0mM isoOMPA (tetraisopropylpyrophosphoramide, Sigma) was used to inhibit 'pseudocholinesterase' (i.e. butyrylcholinesterase) [Silver 1974], acetylthiocholine iodide (Sigma) being the substrate. In the original method [El-Badawi and Schenk 1967], ethopropazine hydrochloride ($2x10^{-5}$M, Sigma) was used as a butyrylcholinesterase inhibitor, however it was found by the author to cause a brown precipitate of copper salts in the reaction medium and subsequent poor staining of the acetylcholinesterase: isoOMPA proved to be a better alternative in subsequent experiments.

An alternative control was provided by the specific inhibition of acetylcholinesterase by the addition of 0.5mM BW284C51 (Wellcome) to the reaction medium [see Silver 1974]. Addition of an alternative substrate, butyrylthiocholine iodide (Sigma) revealed the distribution of butyrylcholinesterase.

An internal control was provided by the red blood cells present in the tissue sections which have been shown to stain specifically for acetylcholinesterase [Silver 1974]. Rat submandibular gland and rat heart were used as positive control tissue. It was found that the cholinesterase remained active for up to 72 hours post mortem.

2.4.2. Glyoxylic acid-induced fluorescence of catecholamines.

The glyoxylic acid technique was utilised to identify and assess the distribution of catecholamines in human lung tissue.

Sections were stained according to the method of Konig [1979]. 10µm sections of fresh frozen tissue were cut on a cryostat at -30°C and placed directly in 1% aqueous solution of glyoxylic acid (Sigma), adjusted to pH 7.4 with sodium hydroxide and containing 0.236M potassium dihydrogen phosphate and 0.2M sucrose. After 30 seconds, the slides were drained and air-dried with cold air from a hair dryer for three minutes before being placed in an oven at 60°C for five minutes. The sections were mounted in 'fluoromount' medium and placed on a hotplate at exactly 80°C for exactly three minutes. The sections were observed using ultra violet microscopy with a Leitz 'D' excitation/suppression filter (355-425nm and 460nm respectively). Positive staining appeared as bright green flourescence. Background autofluorescence was assessed by immersing the sections in the sucrose phosphate solution without glyoxylic acid and processing as usual. To compare structures observed under ultra violet illumination with those seen in the visible range, alternate sections were stained with haematoxylin and eosin (see appendix 3). With experience structures such as blood vessels, bronchial smooth muscle etc. could easily be identified under ultra violet illumination due to the high level of yellow autofluoresence of the collagen and elastin present.

Controls.

Sections stained with glyoxylic acid and sections that had been immersed in the sucrose phosphate solution only (without glyoxylic acid) were coded by an independent worker and observed by the author to assess the accuracy with

which a positive staining reaction could be determined. After examination by the author, the coverslips were removed with xylene and the sections exposed to 0.5% sodium borohydride (Sigma) in 80% aqueous ethanol, which removes the fluorophore, and then re-examined to confirm the presence of a positive reaction in the tissue [Pearse 1969].
Rat heart and submandibular gland were used as positive control tissue.

To distinguish between positive reaction produced by serotonin (5-HT) and other catecholamines (noradrenaline and dopamine), sections were incubated in a glyoxylic acid solution adjusted to pH 3.5 with sodium hydroxide. [Furness and Costa 1975]. Glyoxylic acid at this pH will react with serotonin to produce a yellow fluorophore, but not with noradrenaline or dopamine. It was shown using this method that the positive reaction, observed as bright blue-green fluorescence, in sections of human airway was not attributable to serotonin, and the bright spots of intense yellow fluorescence observed in airway ganglia were also not due to serotonin staining and were therefore assumed to be lipofuscin granules.

Pilot experiments showed that leaving the sections longer than 3 minutes on the hot plate causes them to burn and the background to fluoresce brightly, preventing identification of the positive fluorophore. Tissue sections left to dry outside the cryostat for any length of time before staining yeild negative results and high background autofluorescence. Tissue sections greater than 10u thickness will develop small crystals during the staining process, often yeilding poor results. Processed slides could be stored for up to 2 weeks at $4^{o}C$ in the dark without fading. Tissue could be stored for up to two months at $-70^{o}C$ or 1 month at $-30^{o}C$ before being processed, without losing its reaction. Also, it was found that tissue more than four hours post-

mortem had lost most of its catecholamine content, which could not be restored by preincubation with L-DOPA.

2.4.2.1. Prelabelling experiments.

In order to compensate for atmospheric oxidation of the catecholamines in the human lung specimens, experiments with prelabelling were carried out. Prelabelling with a metabolic precursor of noradrenaline such as L-DOPA theoretically allows the depleted nerve fibres to regain their catecholamine content [Pack and Richardson 1984]. Specimens of lung tissue were incubated at 37°C for half an hour in either Kreb's Henseleit or Weymouths culture medium bubbled with 95% O_2/5% CO_2 (BOC) and containing 1mg/ml of L-DOPA (Sigma). The addition of a small amount (approximately 0.5mg) of ascorbic acid was found to be necessary to prevent oxidation of the L-DOPA. After the incubation period the tissue was rinsed in fresh medium and then blotted dry before being being snap frozen in the usual way. The results of the methods of preincubation are summarised below:-

Sections were coded by an independent worker and the areas of submucosal gland on the sections were examined and assessed semi-quantitatively by the author (see section 2.5.). The mean score (± the standard error of the mean) from the tissue that had been preincubated with L-DOPA and that from the control tissue were 1.92±0.69 and 1.67±0.65 respectively. Comparison using the Mann-Whitney U test showed that the results were not significantly different.

2.4.3. Immunohistochemistry.

Immunohistochemical techniques were applied to stain neuropeptides in human lung tissue. Methods which claim increased sensitivity were applied to attempt to identify neuropeptides in routinely-fixed, paraffin-embedded tissue.

Full details of all the antibodies and reagents used for immunohistochemistry are given in appendix 4.

2.4.3.1. Indirect immunofluorescence.

Benzoquinone-fixed tissue was snap frozen and 10µm sections were cut on a cryostat at -30°C and collected on poly-L-lysine (Sigma) coated slides. The sections were allowed to dry at room temperature for half an hour before rinsing in 0.05M TBS, pH 7.4 containing 2.5% sodium chloride and 0.2% triton-X-100 to reduce the level of background stain by reducing respectively non-specific ionic attraction and non-specific attraction of serum proteins to the tissue [Grube 1980, Hartman 1973]. The primary antibody was applied at the optimum dilution established by prior dilution trials and the sections incubated at 37°C for 30 minutes in a damp chamber. The sections were rinsed 3 times in TBS buffer (as before) for 10 minutes each time, following which they were incubated with the FITC-conjugated secondary antibody (optimum dilution previously determined) for 30 minutes at room temperature. Finally, the sections were thoroughly rinsed in TBS buffer and mounted in TBS/glycerol 4:1 and viewed under ultra violet illumination using a Leitz 'I_2'excitation/suppresion filter (450-490nm and 515nm respectively).

It was found that section staining intensity could be considerably improved

by repeated application of the first layer antiserum, with thorough rinsing (3 x 10 min) between applications [Gu et al 1982]. Pilot experiments showed that the density of VIP-immunoreactivity in the tissue remained constant for up to 24 hours.

To establish the optimum dilution of primary antibody (rabbit anti-VIP, ICN Biomedicals Ltd.), rat lung and gut and human lung tissues were fixed in 0.4% benzoquinone (see section 2.3.2). Cryostat sections were incubated with the primary antibody in a range of dilutions (1:10 - 1:1000). The staining reaction was then assessed against the amount of background, non-specific staining (+ = very little stain, +++ = alot of stain). The results of the dilution trial are summarised below in table 2.

TABLE 2.

Results of the dilution trial of anti-VIP antibody.

Dilution of antibody	+ve stain	Background
1:10	+	+++
1:50	++	+++
1:100	+++	++/+
1:500	++	+
1:1000	+	+

Dilution of second layer FITC-conjugated swine anti-rabbit IgG: **1:100**
Dilution of primary antibody used in subsequent experiments: **1:100**

Controls.

Inhibition control: preabsorption of primary antiserm with 10 nmol/ml synthetic VIP (Sigma).

Negative control: Substitution of primary antiserum with non-immune rabbit serum to same dilution.

2.4.3.2. Avidin-Biotin enhancement.

10µm cryostat sections of benzoquinone-fixed, snap frozen tissue were cut at -30°C. After allowing the sections to dry in room air for no longer than 30 minutes, they were rinsed in TBS buffer (as before) for 5 minutes. The sections were then exposed to avidin (1mg/ml distilled water) for 20 minutes to block endogenous biotin. Following this, the sections were incubated with biotin (0.1 mg/ml distilled water) for 20 minutes to remove the excess avidin. The sections were then incubated with neat normal swine serum for 15 mins.

Following the initial blocking stage the sections were incubated with the diluted primary antiserum for 1 hour in a moist box. After three 5 minute rinses the sections were incubated with the second layer biotinylated antibody for 30 minutes, rinsed 3 times for 5 minutes with TBS buffer and incubated with the third layer FITC-avidin for 30 minutess. Finally the sections were rinsed again 3 times for 5 minutes, mounted in buffered 25% glycerol and observed under ultra violet illumination at 490nm excitation wavelength.

All incubations were at room temperature unless otherwise stated.

The optimum dilution of primary antibody (rabbit anti-substance P, CRB Ltd.) was established as before. The test tissues were guinea-pig trachea (positive control tissue) and human bronchus.

TABLE 3.

Results of the dilution trial of anti-substance P antibody.

Dilution of antibody	Result	Background
1:10	-ve	+++
1:50	+	+++
1:100	+++	++
1:500	+	+
1:1000	-ve	+

Secondary antibody: Biotinylated swine anti-rabbit, **1:500.**

Tertiary antibody: Avidin-FITC, **1:100.**

Controls.

Inhibition control: Preabsorption of primary antiserum with 5 nmol/ml synthetic substance P (Sigma).

Negative control: Substitution of primary antiserum with non-immune rabbit serum at same dilution as primary antibody

2.4.3.3. Peroxidase anti-peroxidase technique.

5µm sections of formalin-fixed, paraffin embedded tissue were dewaxed in xylene and rehydrated in graded ethanols (100, 90 and 70%). Benzoquinone-fixed, 10µm cryostat sections were allowed to dry at room temperature for 30 minutes. The sections were then soaked in buffered detergent (0.2% Triton-X-100 in PBS) and rinsed in PBS. Endogenous peroxidase was blocked by immersing the slides for 30 minutes in a solution of 0.3% hydrogen peroxide in PBS and then rinsing in fresh PBS. Diluted non-immune goat serum (1:10 - 1:30) was applied to the sections for 10 minutes before the application of the first layer antiserum, which was raised in rabbit. The sections were incubated overnight at 4°C in a moist box. After thorough rinsing in PBS buffer (3 x 10min) the second layer antiserum, unconjugated goat anti-rabbit IgG, was applied to the sections for 45 minutes at room temperature. The sections were rinsed again (3 x 5min) in PBS before addition of the third and final layer, the PAP complex. After 45 minutes at room temperature, the sections were rinsed again (3 x 5min) in PBS. The reaction was developed by applying 25% DAB (diaminobenzidine, Sigma) in

PBS containing 50-100µl of 100 volume hydrogen peroxide per 100ml. The reaction was monitored microscopically to determine the optimum reaction time. After development the sections were rinsed in distilled water and counterstained with haematoxylin, dehydrated and mounted (see appendix 3). The tissue retained a positive reaction for NSE and PGP 9.5 for up to 72hrs.

(Because DAB is potentially carcinogenic, the development step was carried out in a fume cupboard and contaminated equipment was neutralised by soaking in concentrated sodium hypochlorite).

The optimum dilution of the primary antibody (rabbit anti-NSE, Dako) was established as before; 10% neutral buffered formalin-fixed and paraffin embedded human pancreas (positive control tissue) and human lung were used as the test tissues.

TABLE 4.

Results of the dilution trial of anti-NSE antibody.

Dilution	+ve stain	Background
1:200	-ve	+++
1:400	+	+++
1:1000	++	++
1:2000	+++	++/+
1:4000	++	+

Second layer antibody: Unconjugated goat anti-rabbit IgG, **1:200**

Third layer antibody: PAP complex, **1:400**

Dilution of NSE used for further study: **1:2000**

Dilution of PGP 9.5 used for comparison of staining: **1:500**

(For details of antibodies and reagents used see appendix 4)

Controls

Inhibition control: non available

Negative control: substitution of the primary layer antiserum with non-immune rabbit serum of the appropriate dilution

2.4.3.4. Immunogold/silver enhancement.

5-6µm sections of 10% neutral buffered formalin fixed, paraffin embedded tissue were cut on a microtome (Reichert-Jung U.K. Ltd.) and collected on poly-L-lysine coated slides. The sections were incubated at 55°C for 10 minutes to ensure section adhesion. The sections were then dewaxed in xylene, rehydrated in graded alcohols and twice rinsed in distilled water for 1 minute. The sections were exposed to Lugol's iodine (1g of iodine in 100ml 2% aqueous potassium iodide solution) for 5 minutes and rinsed in 2.5% sodium thiosulphate until colourless. Following thorough washing in running tap water for 10 minutes the sections were rinsed in TBS (0.05M Tris buffer, pH 7.4 containing 2.5% sodium chloride, 0.2% Triton-X-100 and 0.01% sodium azide) for 5 minutes. The sections were incubated with neat normal goat serum for 10 minutes before application of the first layer primary antiserum (diluted in TBS pH 7.4 containing 0.1% bovine serum albumin, Sigma). After an incubation period of 90 minutes in a moist box, the sections were thoroughly rinsed in TBS pH 7.4 (3 x 10 min) and then in TBS (0.05M, pH 8.2) for 5 minutes. A second blocking step was then carried out with neat normal goat serum for 10 minutes. The second layer gold conjugated antibody (diluted in TBS, pH 8.2 containing 0.8% bovine seum albumin) was applied and the sections incubated in a moist box for 1 hour. Following the incubation the sections were rinsed in TBS buffer rinse for 3 x 10 minutes and in deionised distilled water for 6 x 5 minutes. The silver enhancement

was carried out using a commercially available kit (Janssen). Developing and fixation solutions were made up immediately before the enhancement step. The development of the reaction was monitored microscopically. The sections were then fixed for 2-3 minutes, rinsed in running tap water for 10 minutes and counterstained with haematoxylin, eosin and alcian blue, dehydrated in graded alcohols, cleared in xylene and mounted in DPX (see appendix 3). All incubation steps were carried out at room temperature. The optimal dilution for each antibody was determined by varying the dilutions of both primary and secondary antisera until the maximum contrast between background and positive stain was acheived.

TABLE 5.

Dilution of primary antibody and controls for immunogold/silver enhancement.

Antibody	Optimal dilution	Inhibition control	Control tissue
PGP 9.5	1:500	None available	Human bronchus
VIP	1:1000	10 nmol/ml	Cat trachea
SP	1:500	5 nmol/ml	Rat lung
CGRP	1:1000	1 nmol/ml	Rat lung
NPY	1:500	1 nmol/ml	Rat spine

Secondary antibody: Goat anti-rabbit gold conjugated IgG, 1:40

Negative control: Substitution of primary antiserum with non-immune rabbit serum at same dilution as the primary antibody.

2.4.4. Radioimmunoassay.

Radioimmunoassay was applied to determine the concentrations of VIP and substance P in normal and cystic fibrosis lung.

2.4.4.1. Assay of vasoactive intestinal peptide.

The assay of VIP was carried out using a commercial assay kit (Amersham) containing the radiolabel and antiserum to VIP.

i. Tissue extraction.

Samples of snap frozen human lung were allowed to defrost and chopped into pieces (1mm cubes). The tissue was then boiled in 0.5M acetic acid for ten minutes, (1mg tissue to 10ul acetic acid). The samples were cooled on ice and centrifuged at 2000g for 10 minutes. The supernatants were then applied in the assay.

ii. Standard curve.

400µl of buffer (50mM sodium phosphate, pH 7.2 containing 0.3% bovine serum albumin and 10mM EDTA) was added to each assay tube into which 100µl of label solution was placed ([3-iodotyrosyl-^{125}I]VIP diluted to a concentration of 24,000cpm/ml with assay buffer). The standard solution of VIP (Sigma, 0.4nM in assay buffer) was added to the tubes in the following amounts:

2µl(0.8fmol), 3µl(1.2fmol), 5µl(2fmol), 7µl(2.8fmol), 10µl(4fmol), 15µl(6fmol), 20µl(0.8fmol), 25µl(10fmol), 50µl(20fmol), 100µl(40fmol). 100µl of antiserum (rabbit anti-VIP serum dissolved in 2ml of assay buffer and then diluted to 25ml with assay buffer) was added to every tube except a blank. The

volume in each tube was made up to 800µl with assay buffer, covered and left at 4°C for 6 days.

With each assay, a blank was prepared which consisted of 700µl buffer, 100µl label solution, diluted as above, with no antiserum and no standard solution. The zero solution consisted of 600µl buffer, 100µl label solution, 100µl antiserum solution and no standard solution.

iii. Tissue samples.

To analyse tissue samples, assay tubes were prepared as above, except that instead of adding the standard solution of VIP, 100µl of the tissue extract was added to each tube.

iv. Separation procedure.

0.4g of Norit GSX activated charcoal (BDH) and 0.04g dextran (mol.wt. 60000-90000, BDH) were suspended in 50ml of assay buffer and stirred for 20 minutes at 4°C. 0.5ml of dextran-coated charcoal suspension was added to each assay tube, without letting the charcoal settle. The suspension was centrifuged at 2000g for 10 minutes at 4°C. The supernatant and charcoal were separated and the pellet fractions (free) and supernatant (bound) counted for 200 seconds in a gamma counter (Beckmann Gamma 5500).

The results are counted as % bound: $\frac{\text{counts bound}}{\text{counts bound + counts free}}$ x100

(The standard curve was plotted on log linear paper as % bound against fmoles of VIP per assay tube).

2.4.4.2. Assay of substance P.

i. Tissue extraction.

Tissue extraction was carried out as for the VIP assay.

ii. Standard curve.

40µl of buffer (2M Tris, pH 8.6, Sigma) was added to each sample tube. 50µl of label (Substance P, ^{125}I-labelled with Bolton and Hunters reagent, and diluted to 100,000cpm/ml, Amersham) was added along with 50µl of rabbit anti-substance P antibody at a dilution of 1:8000. Standard solutions of substance P (Sigma) over a range of 0.39fmols - 400fmols were added to the tubes in 100µl aliquots. The volume in each tube was made up to 500µl with buffer, covered and left for approximately 6 days at 4°C. A blank and a zero tube were included with each assay as with the VIP assay.

iii. Tissue samples.

Tissue samples were added to each tube (100µl per tube) instead of the unlabelled substance P.

iv. Separation procedure.

The separation procedure was carried out as for VIP (the buffer used was 2M Tris containing 2.5g/litre gelatin).

2.4.5. Spectrophotometric determination of serum cholinesterase.

The aim of the experiment was to determine the levels of butyryl-cholinesterase in samples of serum from patients from five clinical groups:-

i) symptomatic asthma i.e. recieving daily B-adrenergic therapy, ii) asymptomatic asthma, iii) hyperreactive i.e respond to a methacholine challenge of 4mg/ml with a fall in FEV_1 of greater than 20%, iv) hay fever i.e. atopic and v) healthy controls.

Three ml of phosphate buffer (0.01M, pH 8.0) were placed in a perspex cuvette to which 100ul of DTNB reagent (Sigma) and 50µl of neat human serum were added. The solutions were mixed with a clean pasteur pipette before adding 20ul of substrate (0.075M butyrylthiocholine, Sigma), making the total volume in the cuvette 3170µl. A spectrophotometric (Pye-Unicam PU8600 series) reading was taken at 412nm: this represents absorbance by the reaction medium, the zero level of the spectrophotometer having been set with the phosphate buffer. Substrate was added and readings taken at 1 minute intervals for a total of 5 minutes i.e. during the linear phase of the reaction. All reactions were carried out at room temperature, about 21°C.

Calculation of Rate of Reaction. Since the extinction coefficient of the yellow anion of DTNB is (1.36×10^4) [Ellman 1959], the rates can be expressed in absolute units of moles of substrate hydrolysed per minute per ml of serum:-

$$\text{Rate (moles/min/ml)} = \frac{\Delta \text{absorbance/min}}{1.36 \times 10^4} \times \frac{1}{50/3170}$$

Controls.

To establish the rate of non-enzymic hydrolysis of the substrate, a blank run was carried out where the serum was omitted and then the reaction monitored as before. The rate of non-enzymic hydrolysis was 0.0013 absorbance units per minute at 21°C: the rate quoted by Ellman is 0.0016

absorbance units per min at 25°C [Ellman et al 1961].

As a negative control, reactions were also carried out in the presence of isoOMPA which inhibits butyrylcholinesterase:- 100ul of 0.75mM isoOMPA were added to the phosphate buffer plus enzyme and DTNB reagent and left for half an hour before the substrate was added and the reaction was carried out as before. Complete inhibition of the reaction was observed.

To ensure there was no bias in the measurement of the enzyme activity, the serum samples were coded by an independent worker before being assessed by the author.

2.5. QUANTITATIVE METHODS.

This section describes the methods used to assess the distribution and density of the various neural markers under investigation.

2.5.1. Point counts.

In order for the results to be meaningful, the relative areas occupied by the various effector structures must be known. To assess these a point counting method was employed [Aherne and Dunnil 1982]. First, areas to be assessed were selected as follows:-

Each airway sample was routinely sectioned transversely, producing whole or parts rings of airway. To eliminate bias in the assessment of the areas occupied by the structures of interest in the airway wall, an eyepiece graticule (Graticules Ltd.) with two crossed lines splitting the field of view into quadrants was used. (see fig.2.2). The areas for measurement were therefore determined at low magnification (x10 objective) by the position of the dividing lines over the section. Fields for point counting were then observed at higher magnification (x25 objective) using the lines to position the fields from the surface epithelium, across the mucosa outwards to the edge of the section (fig.2.2). When the section did not include a complete airway ring, sampling lines were taken at the two ends of the incomplete ring, the distance between these lines bisected and a third line of sampling taken at this point (see fig.2.3).

For the point counting, a Wiebel grid was used [Wiebel 1963] (Graticules Ltd.) (fig.2.5). This type of grid eliminates the problem of unidirectional structures such as bronchial smooth muscle, which could introduce bias into the count. The grid contains 30 points and only those grids completely

Fig.2.2 Method of selection of high power fields for point counts

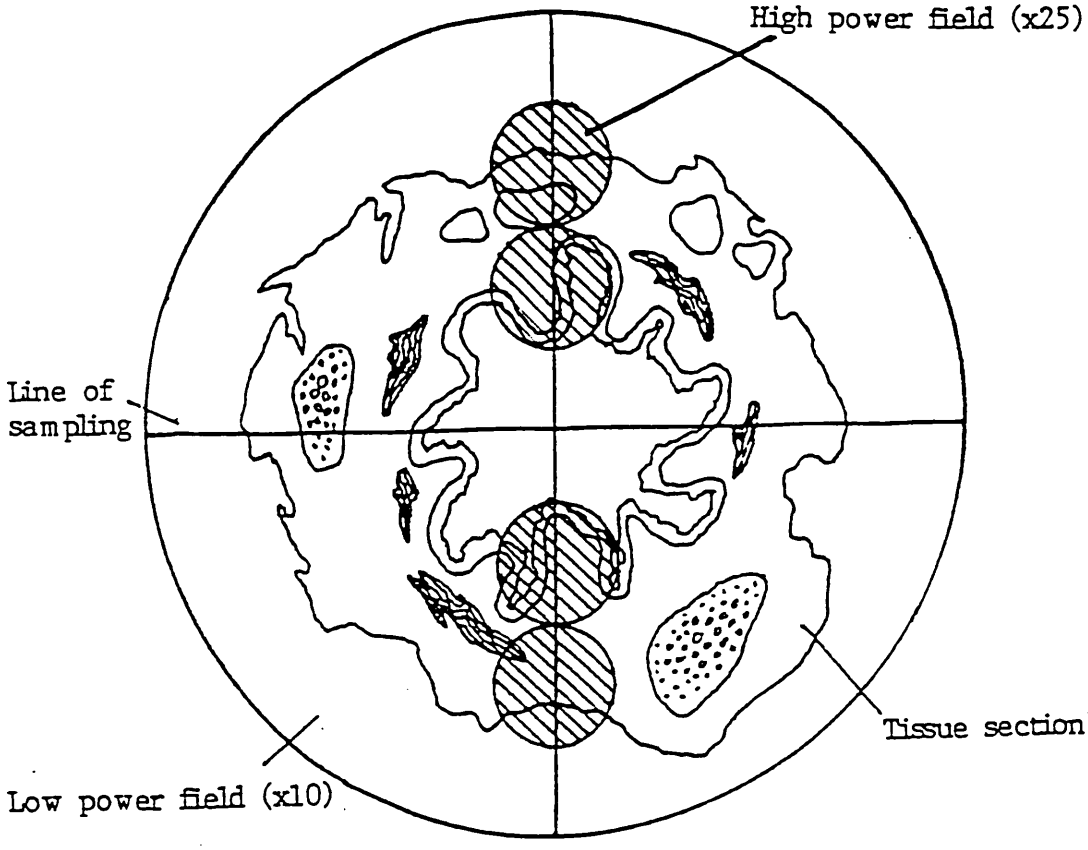
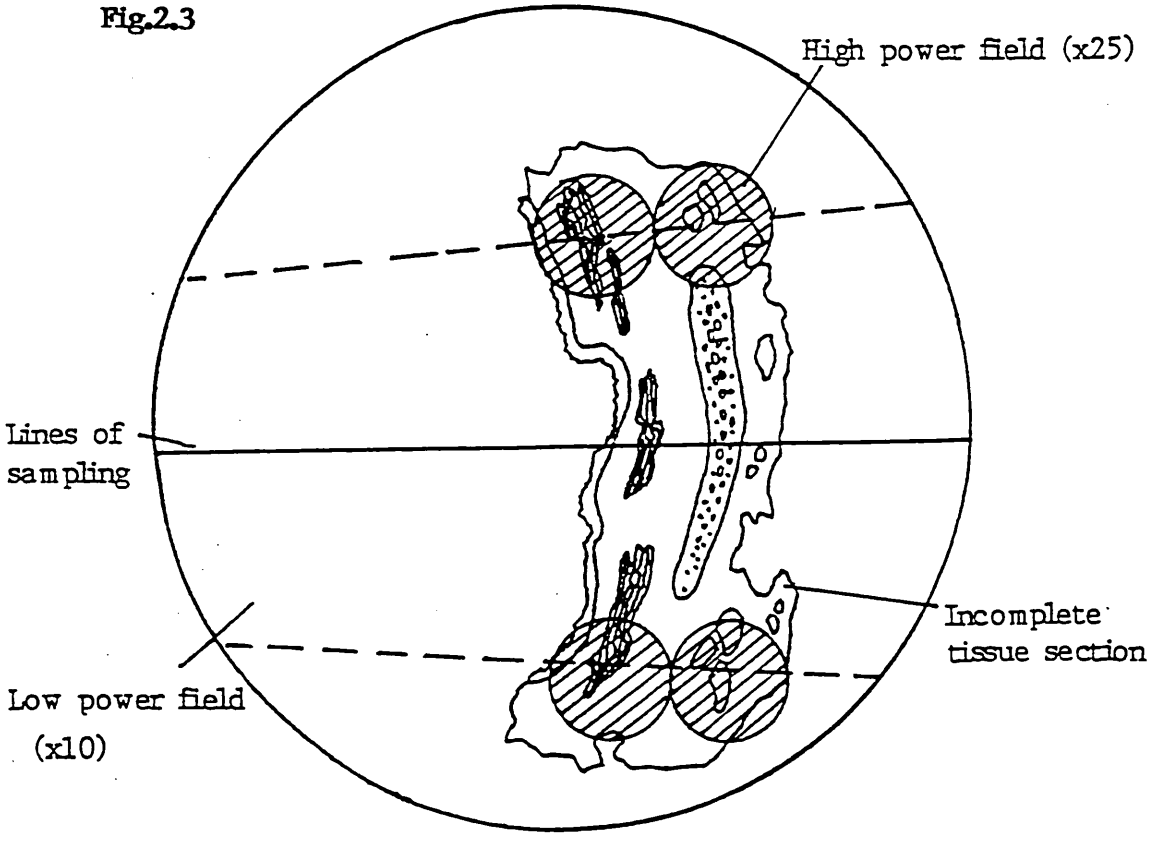


Fig.2.3



occupied by the tissue section were counted. The number of points overlying each of the above mentioned structures was determined: the remainder represents the area of the section occupied by cartilage, connective tissue and artifacts in the section. The relative percentage area occupied by each of the structures in the above categories was calculated from the number of points overlying each, expressed as a percentage of the total.

2.5.2. Methods for evaluation of the number and density of nerve fibres in tissue sections.

The difficulty of evaluating the observations of nerve fibre distribution in tissue sections is of a fundamental nature; how to count a highly branched and weaving structure, often present in bundles where the individual fibres often cannot be discerned. The approach to solving the problem depended on the density of nerve fibres. Fibres present in relatively high density proved practically impossible to count individually, whereas those which appeared relatively infrequently could be counted individually. The methods of evaluation were therefore twofold:

i. Quantitative assessment; counting individual nerve fibres which were infrequently present.
ii. Semi-quantitative assessment of nerve fibres which were present in relatively high numbers.

2.5.2.1. Quantitative assessments; counting individual nerve fibres.

This approach was utilised for two types of assessment:-

i. Counting the number of nerve fibres associated with surface epithelium and expressed per unit length of epithelium.

Fig.2.4. Method of counting nerve fibres within the epithelium

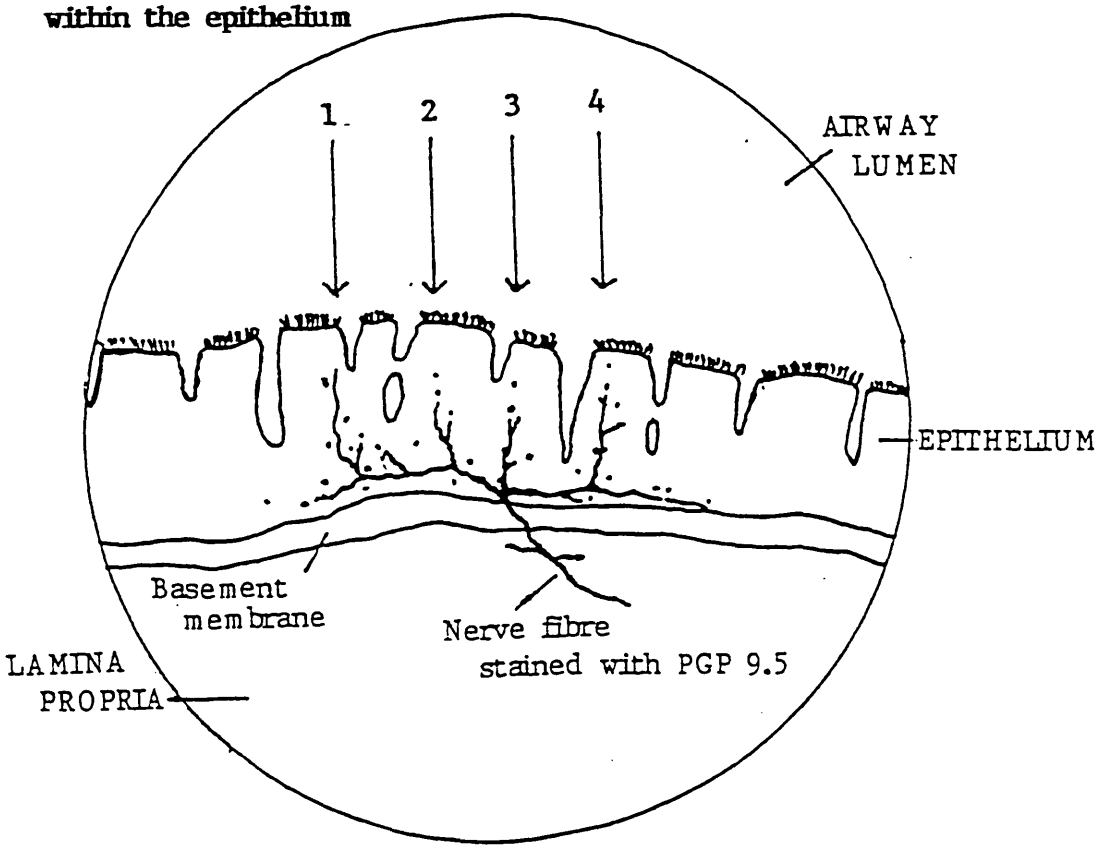
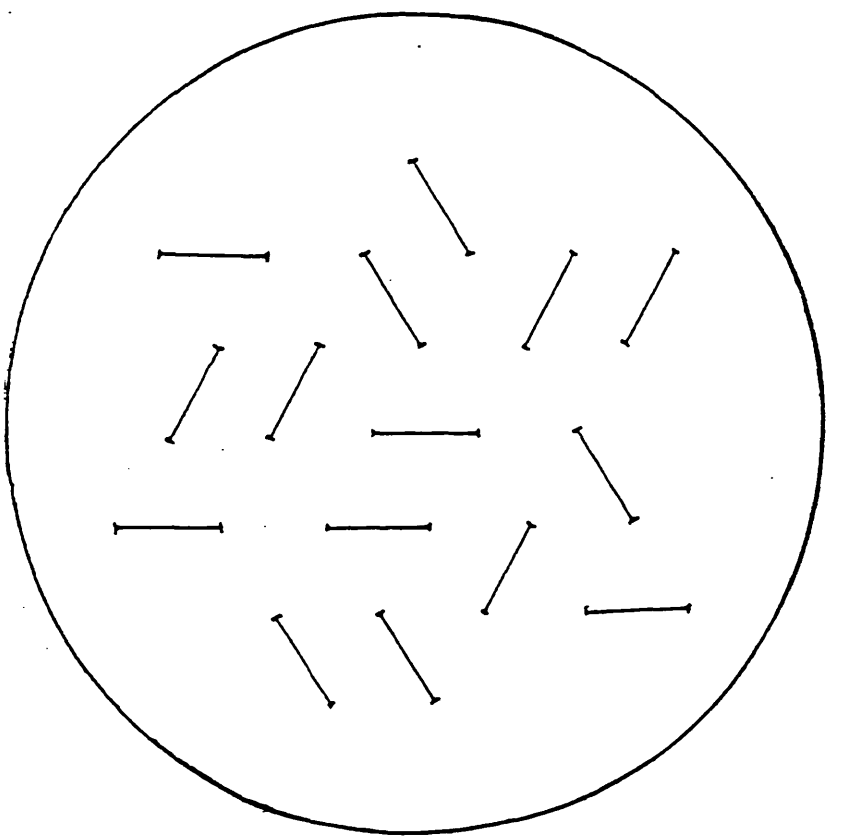


Fig.2.5 Wiebel Grid



Grid designed according to the specifications of Wiebel (1963). The 15 lines connecting the vertices of a regular hexagonal point network ensure an even distribution of the end points.

ii. Assessing the overall distribution of nerve fibres within the tissue section in relation to the percentage area of the section occupied by each effector structure of interest.

i. Counting the numbers of nerve fibres per unit length of epithelium.

In assessing the number of nerve fibres per unit length of epithelium, the first priority was to find an accurate way of measuring epithelial length. The method chosen was the utilisation of a stereological (image analysis) programme on the graphics tablet of an Apple IIe microcomputer (Lavenread Computors Ltd.); an image of a magnetic graphics tablet is superimposed on the image of the section on the back focal plane of the microscope. The observer can then draw around the image of the section, seen through the microscope objective, with an electronic pen on the graphics tablet. The drawing appears on the computer screen and the computer then calculates preset parameters such as area and perimeter length. The computer is calibrated beforehand with an eyepiece measuring graticule (Graticules Ltd.). In this way the length of epithelium is calculated and the number of nerve fibres in that length counted.

The error of repeatibility of measurement using the computer. This was assessed by drawing along the same convoluted length of epithelium 10 times and then calculating the error of repeat measurement. This is expressed as the standard error of the mean calculated as a percentage of the mean. The 'percentage error' calculated in this way for repeat measurements of epithelial length using the computor was 1.5% and therefore the method was deemed a reasonable way of measuring epithelial length.

In assessing the numbers of nerve fibres per length of epithelium, certain criteria were applied to the counting procedure:-

1. In measuring the epithelium only intact lengths were measured i.e. cells attached to the basement membrane.
2. Only nerve fibres on the epithelial side of the basement membrane were counted.
3. Where the nerve fibre branched or represented part of a bundle,only those elements that perpendicular to the length of the basement membrane were counted.

A picture of the counting method is shown in fig.2.4.

The percentage error of repeat counts of nerve fibres made in this way was 2.2%. Variation was assessed by counting the number of nerve fibres in step sections: the percentage error was 5.9%. The slides were all coded by an independent worker before being assessed.

ii. Assessing the overall distribution of nerve fibres within a tissue section.

This was used to quantify those types of nerve that appeared relatively infrequently. The section of interest was scanned for a positive nerve marker. When this was found, its position relative to one of the following structures was noted:-

a. Surface epithelium
b. Submucosal gland
c. Bronchial smooth muscle
d. Nerve bundles/ganglia
e. Lamina propria
f. Blood vessels

Error of repeat measurement. The percentage error of repeat counts of incidences of positive stain made in this way was 3.5%. Again, all the slides were coded by an independent worker before being assessed.

2.5.2.2. Semi-quantitative assessments.

Semi-quantitative assessments were based on a scale of 0-5:-

0 = no nerve fibres observed in the section.

1 = very few fibres i.e. 1 or 2 fibres per section.

2 = few fibres i.e. 1 or 2 fibres per field (viewed at x250 magnification).

3 = moderate numbers i.e. most stuctures innervated by 1 or 2 nerve fibres

4 = numerous i.e. many nerves fibres but not necessarily supplying every structure.

5 = Very numerous i.e. every structure that is innervated is positive for the particular nerve type being assessed.

Repeatability of semi-quantitative assessments.

a. Observor error. The scale was determined by the author and sample slides graded accordingly. These slides were then assessed by an independent worker and the results of the grading by the author compared with those by the other worker were not significantly different (Mann-Whitney U test).

b. Repeatability of measurement. Sections coded by an independent worker were assessed by the author on two successive days to evaluate the repeatability of the assessment. The results were not significantly different (Mann Whitney U test).

c. Variation between samples from one patient and between patient groups.

A measure of the relative variation within groups of patients and within samples from the same patient is included as part of the analysis of variance of the semi-quantitative assessments for each type of nerve marker, which is presented in chapter 3; results.

Three sections of each sample were used for future assessments, this decision being based on making the best economy of the limited supplies of diseased tissue.

2.6. STATISTICAL ANALYSIS.

Statistical analysis was carried out on 2 different computors.

i. An Apple Europlus II microcomputor (Lavenread Computors Ltd) using a programme written by Bruce Land; Interstat 1979 (Serendipity Systems Inc.). This was used for the Mann-Whitney U test and 2-way analysis of variance.

ii. A Tandon PCA computor system with hard disk (Tandon Computor Co.) using a 'Statgraphics' programme (Statistical Graphics Corporation). This was used for the Spearman's rank correlation coefficient, the Kolmogorov-Smirnov 2 sample test and the Mann Whitney U test).

These programmes were judged to be acceptable by a professional statistician, Miss M. Rehan.

2.6.1. Levels of Significance.

The statistical probability value (p-value) at which the null hypothesis (i.e. no difference between the groups) was accepted or rejected was 0.05 (i.e. 5%). Therefore, p values of 0.05 or less were taken as an indication of a significant difference between the groups.

Three levels of significance were chosen, **0.05, 0.01** and **0.001.**

2.6.2. Distribution of data and appropriate tests of significance.

The statistical test appropriate to the data was determined after inspection of the frequency distribution of the raw data.

2.6.2.1. Point counts.

The number of sections in each group was too small to establish whether the distribution of the data was normal. Therefore, in assessing the difference between groups in the % areas occupied by different effector structures in the airway wall, the Mann-Whitney U test was employed. This is a 'non-parametric' test that does not necessitate any assumptions about the distribution of the data. This test combines and ranks the data from two samples. The ranks are then summed over all the observations in each sample and a statistic is calculated to compare the average ranks.

2.6.2.2. Quantitative assessments: SP- and CGRP-positivity in tissue sections and PGP 9.5- positive nerves in surface epithelium.

The frequency distribution of individual nerve fibres in association with different effector structures within the tissue section, did not approximate to any standard type of distibution. The distribution was consistently positively curtailed with a strong positive skew. The positive skew was caused by the presence of a few very high scores. The test applied to this data was the Kolmogorov-Smirnov 2 sample test, which tests whether the two samples come from the same distribution by calculating the maximum distance between the cumulative distributions for the two samples. If the distance is large enough, the null hypothesis that the two distributions are the same is

rejected. This comparative method is not disturbed by occasional very high values as were present in the data from the quantitative analysis of the distribution of SP- and CGRP- positive nerves.

In assessing the differences between different groups in the numbers of nerve fibres stained with PGP 9.5 in the epithelium, the Mann Whitney U test was employed because it does not rely on distributional assumptions made about the data and is therefore not invalidated by small sample sizes.

2.6.2.3. Semi-Quantitative assessments: distribution of cholinesterase, VIP, catecholamines, tyrosine hydroxylase and NPY.

These data generally approximated to a normal (Gaussian) distribution but were occasionally skewed either positively or negatively i.e. the median value did not always approximate to the mean value. However, the variance was approximately equal for each group. The analysis of variance was calculated according to how many parameters were to be tested. To analyse the differences between groups and within groups the 2-way analysis of variance was carried out. Differences between airway levels and those between groups and within groups were identified by a hierarchical analysis of variance. The variation between individual samples is reflected in the 'residual' component of both types of analysis. The 2-way and hierarchical analysis of variance assume a normal distribution for the data and an approximately equal variance within groups. The data was deemed by Miss M Rehan to be suitable for this type of analysis, which is fairly insensitive to slight non-normality. Differences observed between groups were confirmed using the Mann Whitney U test. Data tested in this way yielded the same result as using the analysis of variance for differences between groups.

2.6.2.4. Correlation of data.

In correlating the percent area of the tissue section occupied by submucosal gland and the corresponding semi-quantitative score for VIPergic innervation, Spearman's rank correlation coefficient was used. The Spearman procedure sums the squared disagreements between all pairs of data and calculates a relative measure of disagreement. The procedure uses the ranks of the data rather than the numeric values themselves and is therefore suitable for data assessed on a grading system. The coefficient is scaled to fall between -1 and +1. Values closer to zero indicate no correlation between the sets of data.

2.6.2.5. Radioimmunoassay.

Values included one uncharacteristic high score (level of VIP in case 321). These results were therefore compared by using the Kolmogorov-Smirnov 2 sample test.

2.6.2.6. Serum cholinesterase determinations.

Mean values for the rate of hydrolysis of substrate (moles per min per ml serum) for each group were compared using the Mann Whitney U test.

CHAPTER 3.

RESULTS

Samples of human lung tissue were stained for neural markers using cholinesterase histochemistry, glyoxylic acid-induced fluorescence of catecholamines and immunohistochemsitry of peptides on fresh frozen and benzoquinone fixed tissue, and on sections of formalin-fixed paraffin embedded tissue in retrograde studies. The distribution of these markers was primarily assessed in a qualitative fashion in normal lung. In comparing the distribution of neural markers at different airway levels and in disease, the results were assessed quantitatively where the neural markers were infrequently present (e.g. counting the number of nerves per mm epithelial length) and semi-quantitively, using a ranking system of 0-5 to assess the relative densities of neural markers present in large numbers (e.g. acetylcholinesterase and VIP). The disease groups comprised asthma and cystic fibrosis, and in addition to the normal control tissue there were two groups of disease control tissue; bronchiectatic tissue, and 'young' tissue from a group of patients with various conditions e.g. chronic bronchitis, congenital emphysema and lung cysts, with an average age of 28. Point counts were made to determine the relative area on the section occupied by each effector structure. Additional measurements were made of i) the levels of VIP and substance P in normal and CF tissue by radioimmunoassay and ii) the activity of butyrylcholinesterase in normal and asthmatic serum.

3.1. QUALITATIVE ASSESSMENTS: NORMAL LUNG.

3.1.1. Overall distribution of nerve fibres: staining for PGP 9.5 and NSE.

The overall distribution of nerve fibres was delineated by the neural markers NSE and PGP 9.5 in paraffin sections of formalin-fixed human airway (from 7 patients, i.e. n=7), and cryostat sections of fresh frozen human airway (from 4 patients, i.e. n=4).

Sections stained using the PAP technique showed that PGP 9.5 staining was less diffuse and more specific than that of NSE. NSE-positivity was situated in the cytoplasm of submucosal gland cells and on cilia, whereas the staining of PGP 9.5 was much more specific and largely confined to nervous tissue, although some slight staining of cilia occurred. PGP 9.5 was therefore utilised as a general nerve marker for future studies. Furthermore, staining with the immunogold/silver enhancement technique yielded a more intense reaction product than the PAP technique and provided better contrast between the specific stain and background, and between the specific stain and the haematoxylin, eosin and alcian blue counterstains.

Sections stained for PGP 9.5 revealed a generous innervation throughout the airway wall. Thick nerve bundles and ganglia penetrated the submucosa: the density and thickness of these nerves declined with successive airway generations. The majority of ganglia were located extrachondrially and in serial sections of the fresh frozen material, stained positively for acetylcholinesterase. The nerve bundles leaving the ganglia were located both extra- and intrachondrially: many appeared in cross section in the lamina propria indicating that nerve bundles run longitudinally along the airways, branching to form fine networks between the submucosal glands and the epithelium. Fine fibres from this network supplied the bronchial smooth muscle with a rich innervation. The epithelium received a variable supply of fine nerve fibres: occasional fibres pierced the basement membrane from the lamina propria and branched out through the epithelium. There was no consistent correlation between the nerve fibres and goblet or ciliated cells, but neuroendocrine cells were sometimes observed in close association with fine varicose fibres (see Plate 5, fig.15). Bundles of nerve fibres and smaller ganglia were associated with submucosal glands, and from these, fine nerve fibres penetrated the glands (see Plate 5, fig.16). The serous and

mucous components of the glands (differentiated by staining alternate sections with the alcian blue/periodic acid-Schiff reaction, see appendix 3), were innervated in approximately equal proportions. Nerve fibres were identified around both pulmonary and bronchial blood vessels but none were seen in alveolar walls.

3.1.2. The distribution of Substance P.

Substance P-immunoreactivity in normal human lung was assessed by the avidin-biotin enhancement technique in benzoquinone-fixed, snap frozen tissue (n=6), and by the immunogold/silver enhancement technique applied to formalin-fixed, paraffin-embedded tissue (n=7).

Of the 6 cases stained by the avidin-biotin technique, only two showed immnunoreactivity for substance P (cases 332 and 204). The reaction was confined to the epithelium where fine varicose fibres were observed. In the specimens stained by the immunogold/siver enhancement technique, fine varicose fibres appeared sporadically in the epithelium (see Plate 4, fig.12) but in fewer numbers than those staining for PGP 9.5. Airway ganglia and nerve bundles were consistently positive for substance P. Punctate staining for substance P was seen between the nerve-cell bodies of the ganglia and in fibres running within the nerve bundles leaving the ganglia. Substance P was only rarely found around blood vessels and in bronchial smooth muscle. No substance P was found in submucosal gland.

3.1.3. The distribution of calcitonin gene-related peptide (CGRP).

CGRP-immunoreactivity was assessed using the IGSS technique applied to sections of formalin-fixed, paraffin-embedded 'normal' human lung (n=7).

CGRP-immunoreactivity was located very occasionally in fine varicose fibres in the epithelium and also in occasional neuroendocrine cells (see Plate 4, fig.13). These fibres were similar to those staining for substance P. The number of neuroendocrine cells staining for CGRP was much less than the number staining for PGP 9.5. Airway ganglia were consistently positive for CGRP, again in punctate stain between the nerve cell bodies (see Plate 5, fig.17) and in fibres within large nerve bundles leaving the ganglia. CGRP was located in submucosal gland of one case (189) and was rarely present in the bronchial smooth muscle and around arterioles. Overall, the distribution of CGRP was very similar to that of substance P.

3.1.4. Cholinesterase histochemistry; the distribution of cholinergic nerve fibres.

The distribution of cholinergic (acetylcholinesterase (AchE)-positive) nerve fibres was examined using fresh, snap frozen 'normal' human lung, (n=13).

Very strong positive staining for AchE was observed in nerve bundles and ganglia similar to those staining for PGP 9.5. (see Plate 1, fig.1). Positive staining was also observed around submucosal gland acini in close association with both serous and mucous cells (determined by staining alternate sections with AB/PAS appendix 3) (see Plate 1, fig.3). In the bronchial smooth muscle, a rich supply of stongly staining AchE-positive fibres was observed

(see Plate 1, fig.2). Many small AchE-positive fibres and occasional bundles of nerve fibres were observed in the lamina propria, mostly in cross section and somtimes running close to the basement membrane (see Plate 6, fig.18). However, no fibres were observed in the epithelium. The majority of vascular innervation was present around arterioles. Fine AchE-positive periarteriolar fibres were occasionally observed at airway levels I-III (see Plate 1, fig.4). In general, the density of innervation of all structures appeared to decrease with increasing airway generation. No AchE-positive nerves were identified in alveolar walls. AchE inhibitor (BW284C51) eliminated all nerve fibre staining.

The distribution of butyrylcholinesterase (BuchE) was determined by the inclusion of butyrylthiocholine iodide and BW284C51 in the incubation medium. This revealed diffuse red-brown staining in the bronchial smooth muscle, the density of which increased with increasing generations of airway (see Plate 6, fig.19). The diffuse stain was also observed in alveolar walls and faint staining was present in some larger pulmonary arteriole walls. This BuchE staining was not observed elsewhere and was therefore confined to bronchial and vascular smooth muscle. Most of the stain was removed by the inclusion of iso-OMPA in the medium,(see Plate 6, fig 20)

3.1.5. The distribution of VIP-like immunoreactivity.

The distribution of VIPergic nerves was determined by the indirect immunofluorescence technique applied to benzoquinone-fixed, snap frozen 'normal' lung (n=6) and by the immunogold/siver enhancement technique applied to formalin-fixed, paraffin-embedded 'normal' lung (n=7).

These techniques yielded similar results, except that in general, fewer fibres were observed in the paraffin sections than in the benzoquinone fixed tissue.

VIPergic nerves were observed in high densities around submucosal gland acini (see Plate 3, fig.8) and in bronchial smooth muscle (see Plate 3, fig.9). VIP-immunoreactivity was also observed in high density in ganglia (see Plate 8, fig.28) which were positive for acetylcholinesterase by serial section. Both pulmonary and bronchial arterioles received consistent VIPergic innervation (Plate 8, fig.29). Many fibres, some in bundles, were observed in the lamina propria. Although some were very close to the epithelium, none was observed within the epithelium. Overall, the distribution of VIP-immunoreactivity was very similar to that of AchE.

3.1.6. Catecholamine fluorescence.

The distribution of catecholamines was studied in fresh, snap frozen 'normal' human lung (n=23).

Since glyoxylic acid reacts with both noradrenaline and dopamine to produce the fluorophore, 3,4 dihydroisoquinoline [Axelsson et al 1972], the catecholamine-positive fibres observed in the present study are henceforth referred to in the text as 'aminergic'. Overall, the distribution of aminergic nerves appeared to be less dense than that of the cholinergic supply. Fine varicose nerve fibres were observed around submucosal gland acini (see Plate 2, fig.5) and occasionally 'en passage' through connective tissue and laminal propria. Again no innervation of epithelium was observed. Innervation of the bronchial smooth muscle was observed in 13 of the 23 cases examined. In those cases that were positive, long beaded nerve fibres ran longitudinally through the smooth muscle (see Plate 2, fig.6 and Plate 7, fig.22) and were

observed down to the level of subsegmental airways, although they were extremely sparse at this site (see Plate 7, fig.23). The possibility that these fibres were innervating blood vessels present in the smooth muscle was discounted by close examination of serial sections stained with haematoxylin and eosin. Small bronchial arterioles recieved a rich supply of aminergic fibres in excess of those observed to stain for acetylcholinesterase (see Plate 7, fig.21). Larger pulmonary arterioles were only occasionally innervated. Positive staining for catecholamines was often observed as bright spots of positive fluorescence in ganglia (see plate 2, fig.7), the serial sections of which stained strongly for acetylcholinesterase. Positive staining was not observed in the nerve bundles leaving the ganglia. Bright spots of intense yellow fluorescence were also observed in the ganglia. These were not removed by sodium borohydride treatment and are therefore probably lipofuscin granules.

3.1.7. Tyrosine hydroxylase: an alternative marker for aminergic nerves.

The distribution of aminergic nerves, as determined by the application of antibodies to the precursor enzyme tyrosine hydroxylase, was determined in formalin fixed paraffin embedded 'normal' human lung (n=7).

The distribution of tyrosine hydroxylase was very similar to the distribution of catecholamines as revealed by the glyoxylic acid technique. Again the distribution of aminergic fibres appeared to be sparse in comparison with the cholinergic supply. Small fibres positive for tyrosine hydroxylase were observed in association with the submucosal gland in similar densities to those observed when the tissue was stained for catecholamine fluorescence. Small arterioles stained strongly for tyrosine hydroxylase (see Plate 8,

fig.25). Large pulmonary arteries also displayed staining around the vascular smooth muscle, although the stain was less dense and of a more diffuse nature than that observed around the smaller bronchial arterioles. Very occasional fibres were observed in the bronchial smooth muscle (BSM, see Plate 8, fig.24). However strong, diffuse staining was present over the smooth muscle fibres, making identification of individual fibres difficult. Positive staining was also observed in the airway ganglia and nerve bundles, but was diffuse rather than punctate as seen with the glyoxylic acid staining (see Plate 8, fig.24). No staining was observed in the epithelium, but was very occasionally present in the lamina propria.

3.1.8. The distribution of NPY.

The distribution of NPY-positive nerve fibres was determined by the application of the immunogold/silver enhancement technique to formalin-fixed and paraffin-embedded normal human lung (n=7).

NPY-immunoreactivity was observed mainly in bronchial smooth muscle (see Plate 8, fig.26) and around arterioles (see Plate 8, Fig.27). The fibres in smooth muscle were more frequent than aminergic fibres (stained with either glyoxylic acid or tyrosine hydroxylase), but not as numerous as cholinergic or VIP-ergic fibres. Both bronchial and pulmonary arterioles were consistently well innervated. Occasional fibres were noted in the lamina propria. However, the epithelium was negative, as was the submucosal gland. Occasional punctate stain was observed in the ganglia and appeared to be distributed in a similar fashion to the catecholamines, stained by glyoxylic acid-induced fluorescence.

PLATE 1: Cholinesterase histochemistry

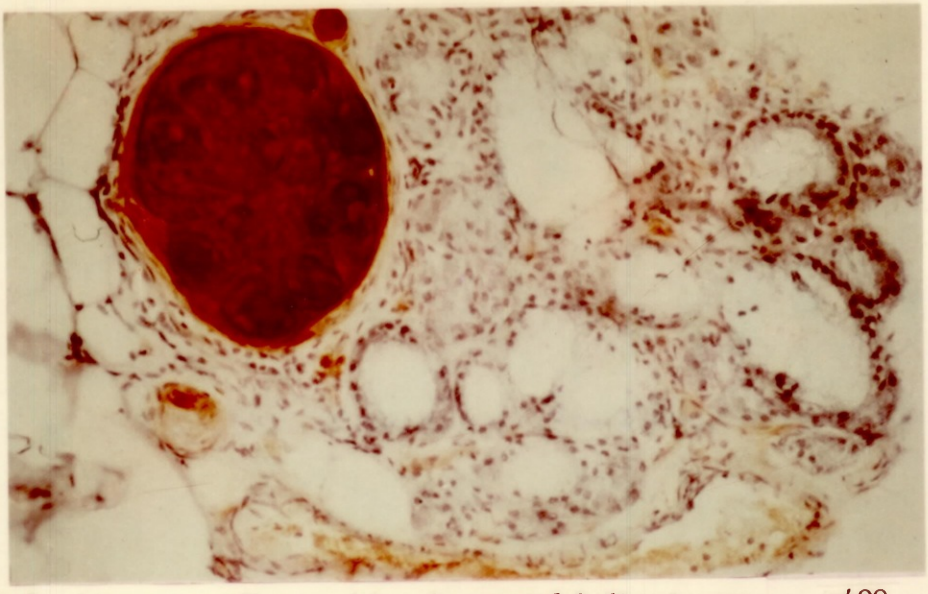


Fig.1 Airway ganglion positive for acetylcholinesterase x400

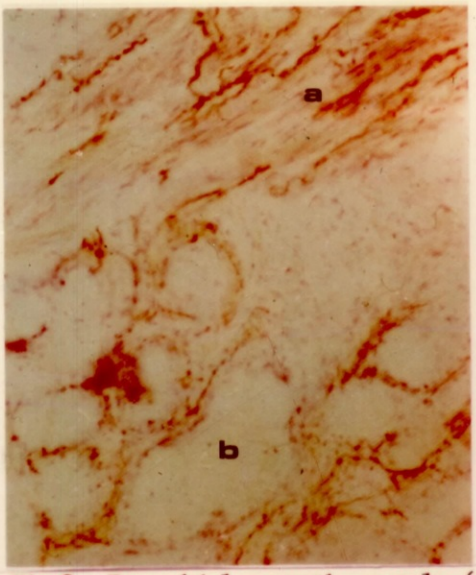


Fig.2 Bronchial smooth muscle (a) and submucosal gland (b) x400

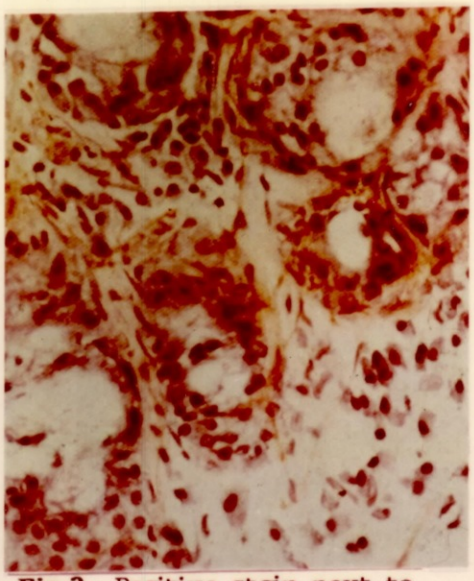


Fig.3 Positive stain next to gland acini x1000

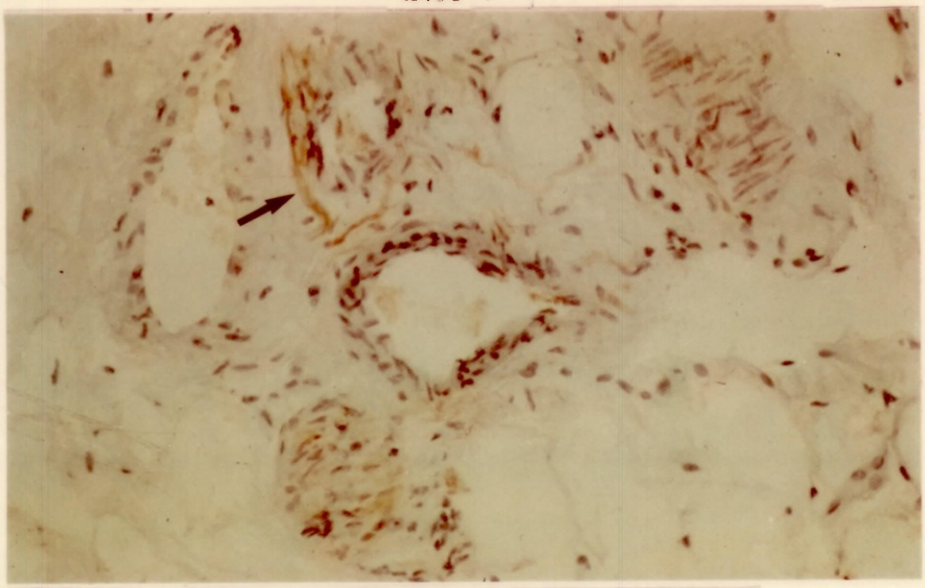


Fig.4 Sparse presence of positive stain in association with arterioles x1000

PLATE 2: Glyoxylic acid-induced fluorescence of catecholamines

Fig.5 Adrenergic nerves around submucosal gland acini x1600

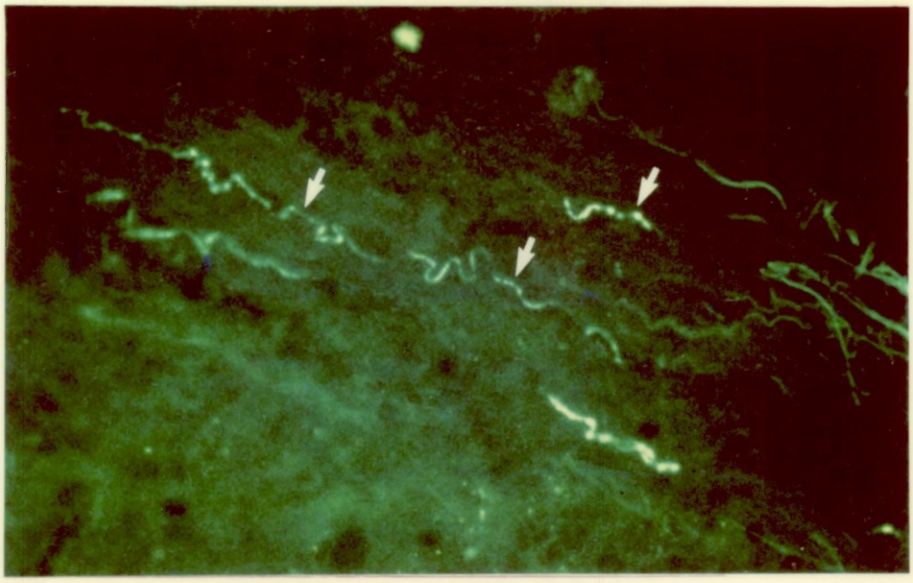


Fig.6 Adrenergic nerves in bronchial smooth muscle x400

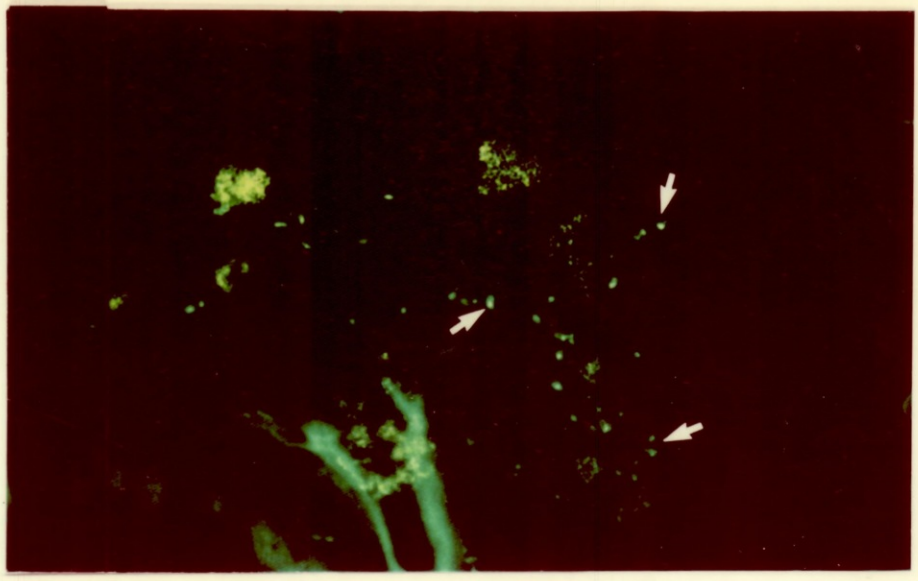


Fig.7 Adrenergic-positive stain in airway ganglia x1000

PLATE 3: Indirect immunofluorescence

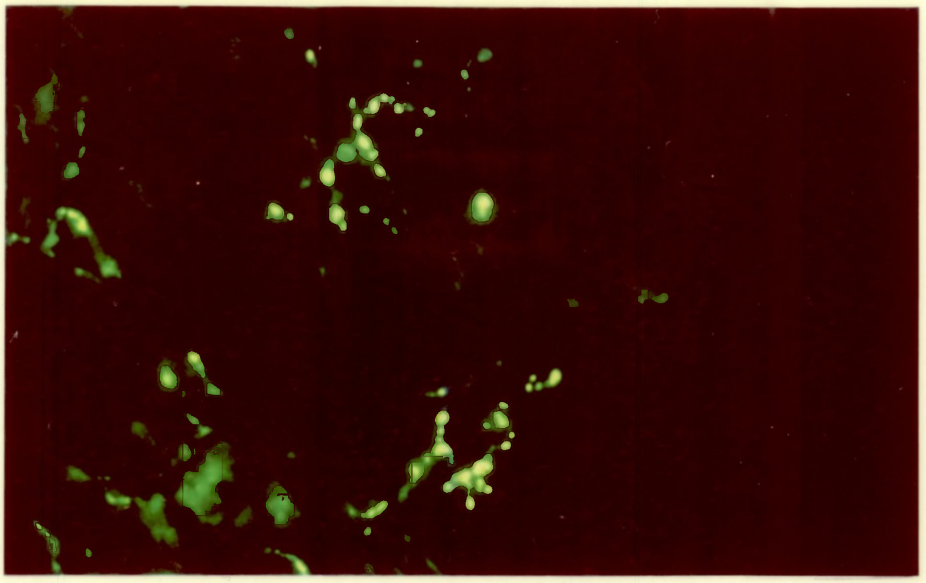


Fig.8 VIP in association with submucosal gland acini x1000

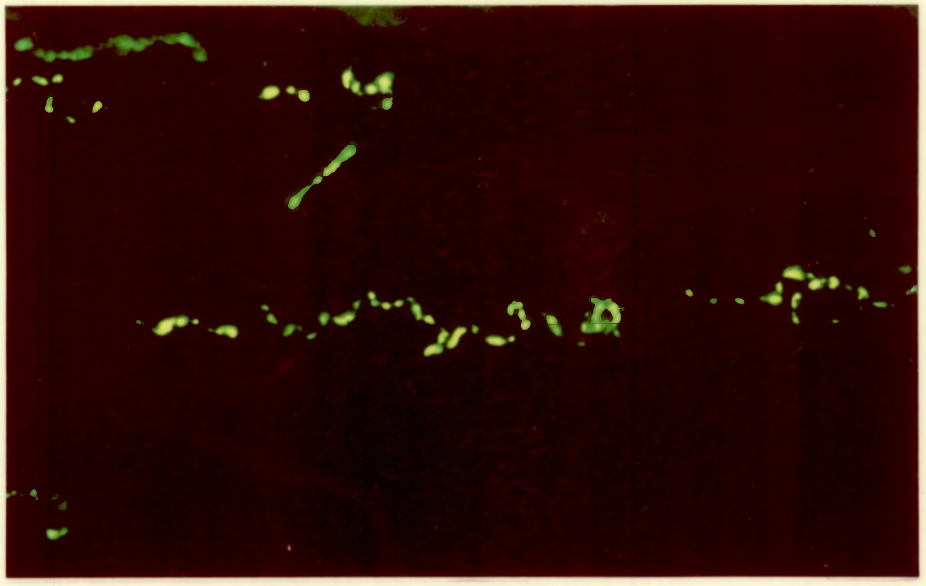


Fig.9 VIP in bronchial smooth muscle x1000

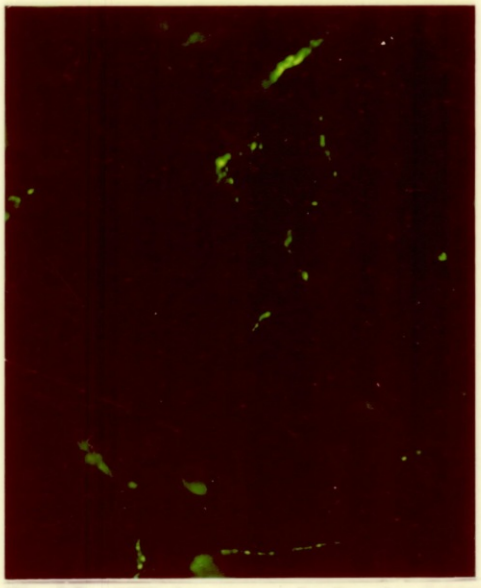


Fig.10 VIP in normal gland x1000

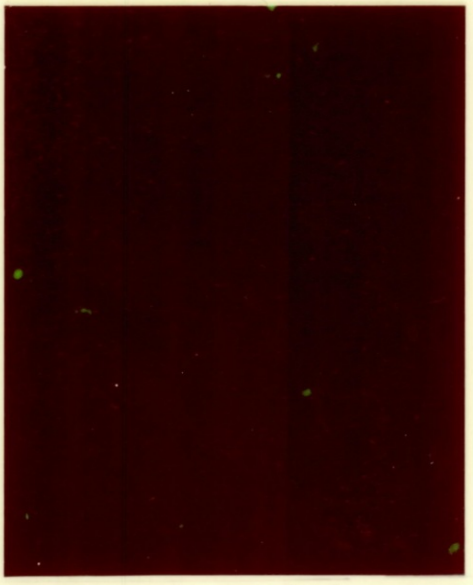


Fig.11 VIP in CF gland x1000

PLATE 4: Immunogold/Silver enhancement

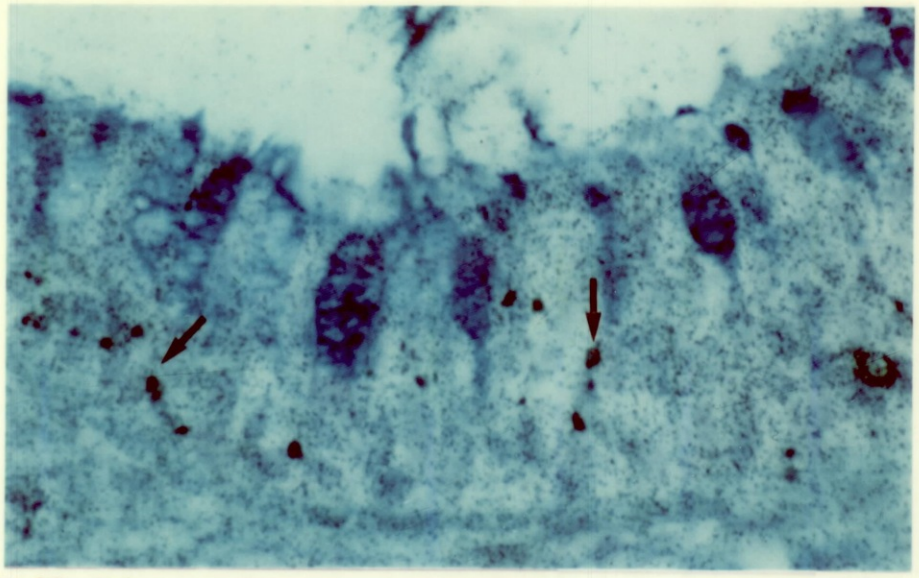


Fig.12 Substance P in airway epithelium x1600

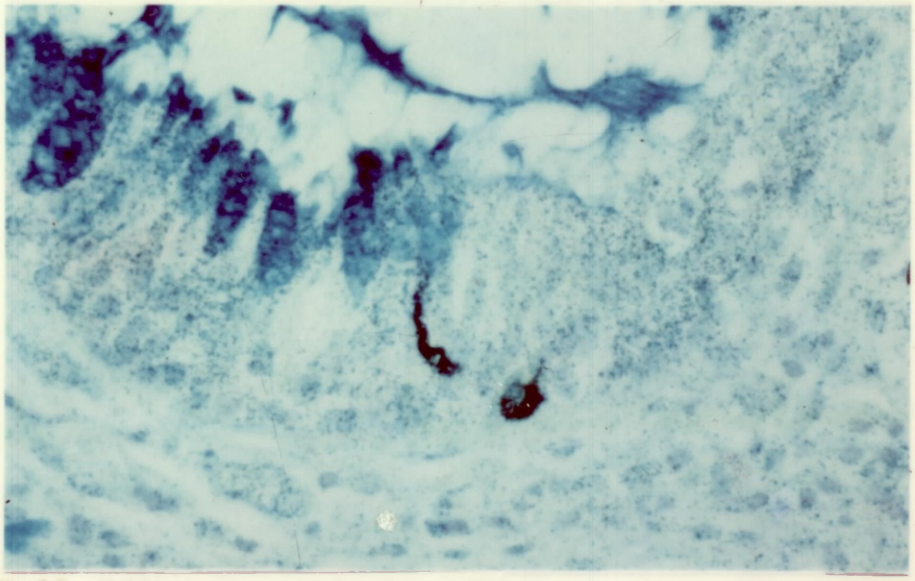


Fig.13 CGRP in neuroendocrine cell x1600

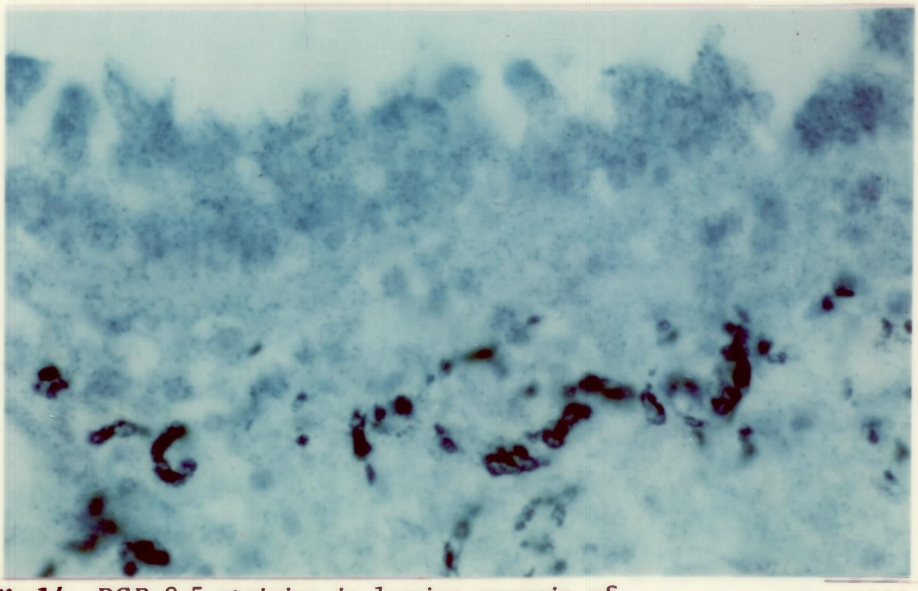


Fig.14 PGP 9.5 staining in lamina propria of asthmatic airway x1600

PLATE 5: Immunogold/Silver enhancement

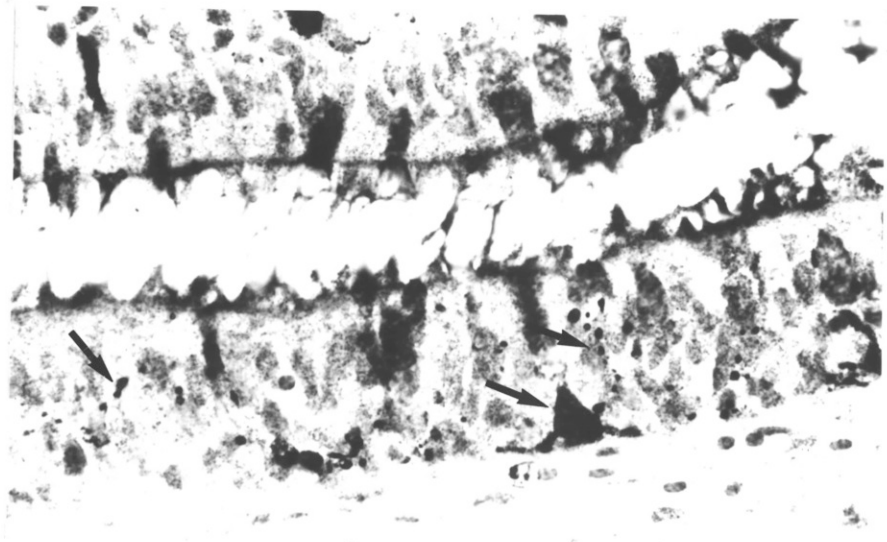


Fig.15 Staining for PGP 9.5 in airway epithelium x1600

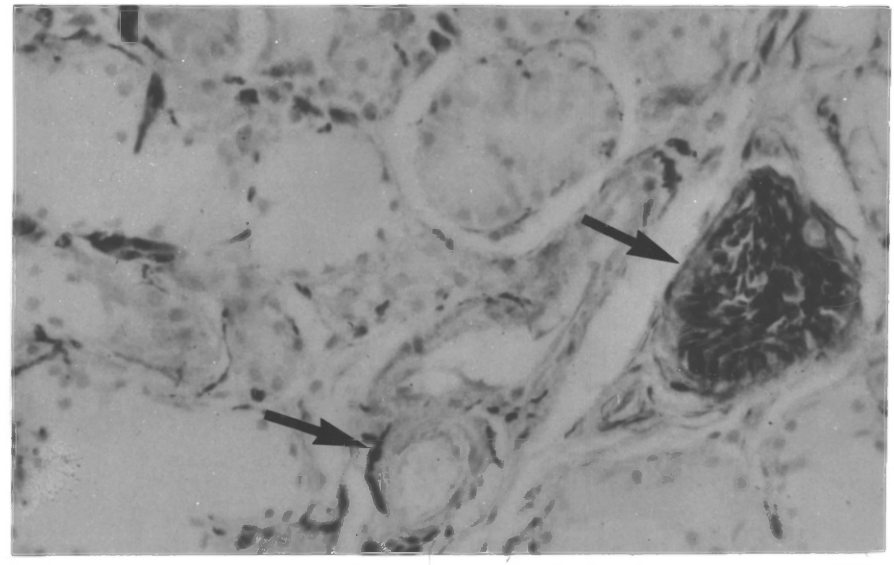


Fig.16 Staining for PGP 9.5 in submucosal gland x800

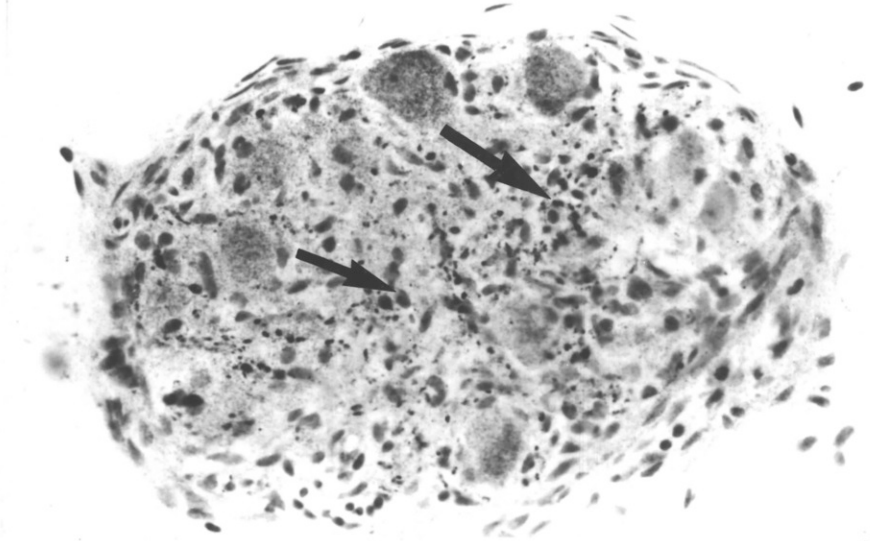


Fig.17 CGRP in airway ganglion x1000

PLATE 6: Cholinesterase histochemistry

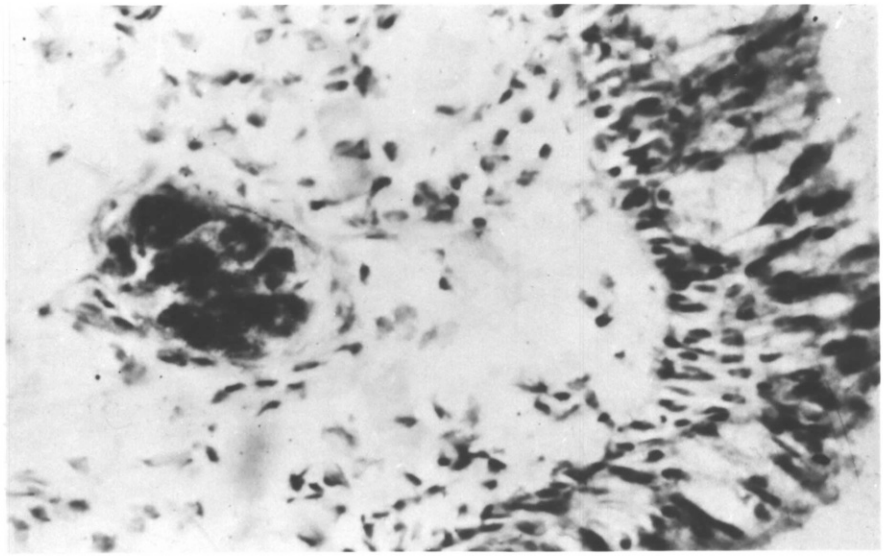


Fig.18 Acetylcholinesterase-positive nerve bundle in lamina propria x1600

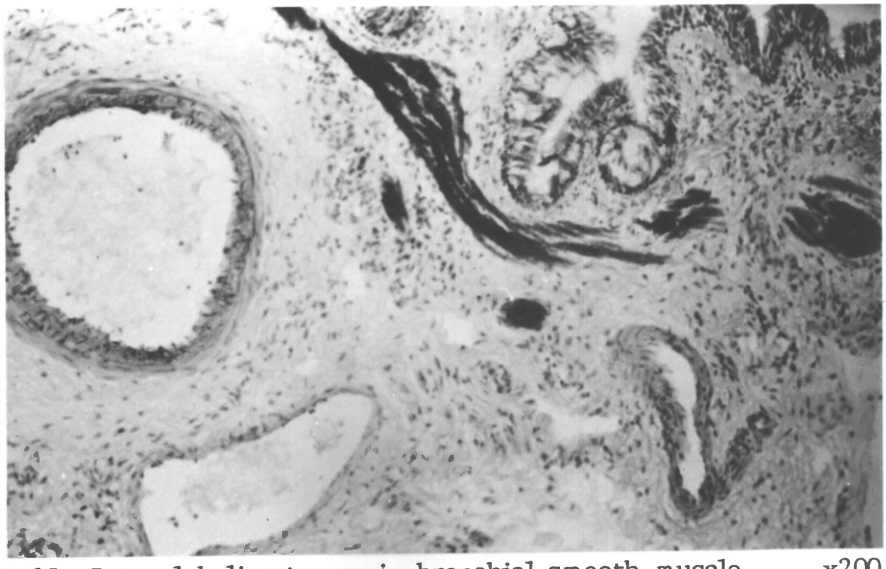


Fig.19 Butyrylcholinesterase in bronchial smooth muscle x200

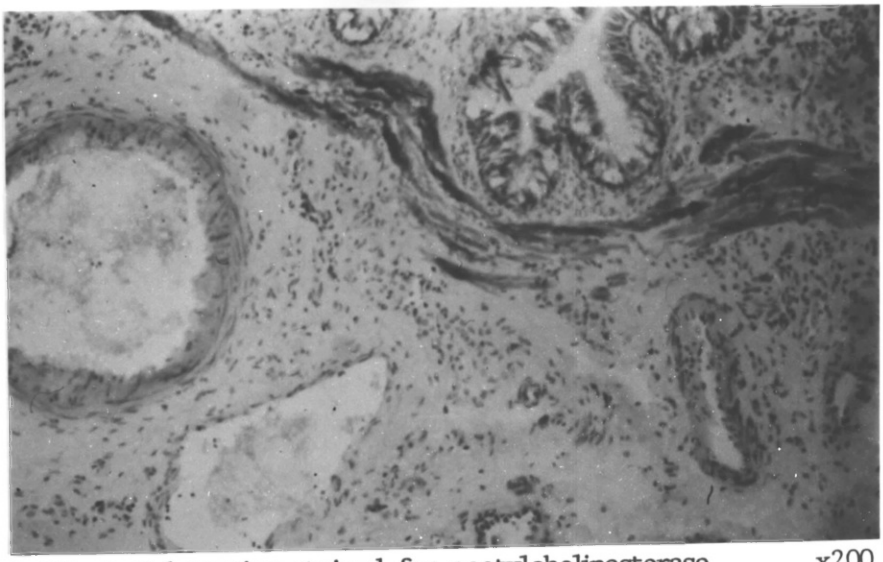


Fig.20 Serial section stained for acetylcholinesterase x200

PLATE 7: Glyoxylic acid-induced fluorescence of catecholamines

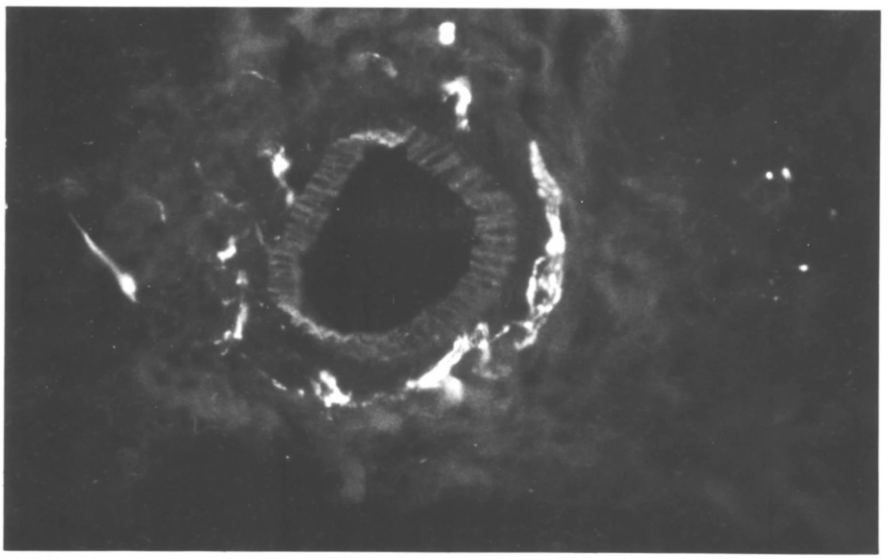


Fig.21 Adrenergic nerves around an arteriole x800

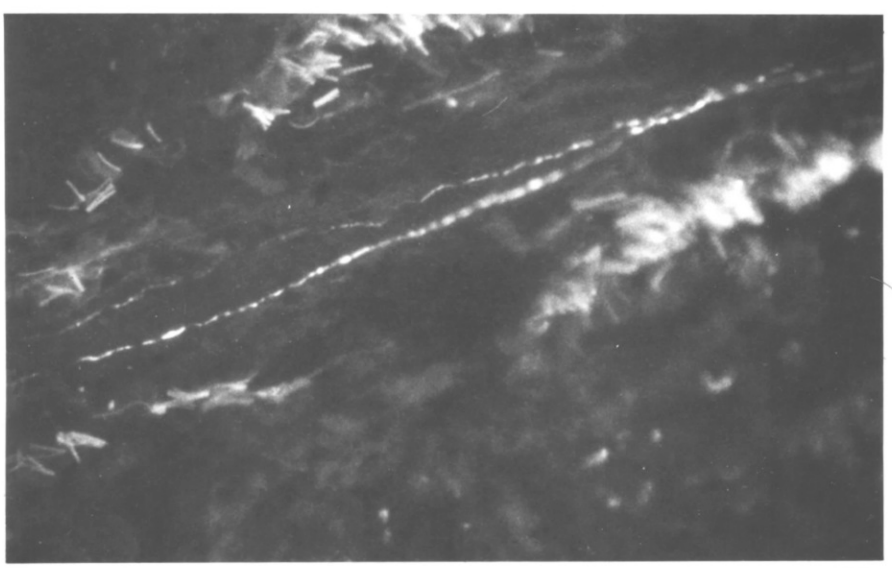


Fig.22 Adrenergic nerves in bronchial smooth muscle x400

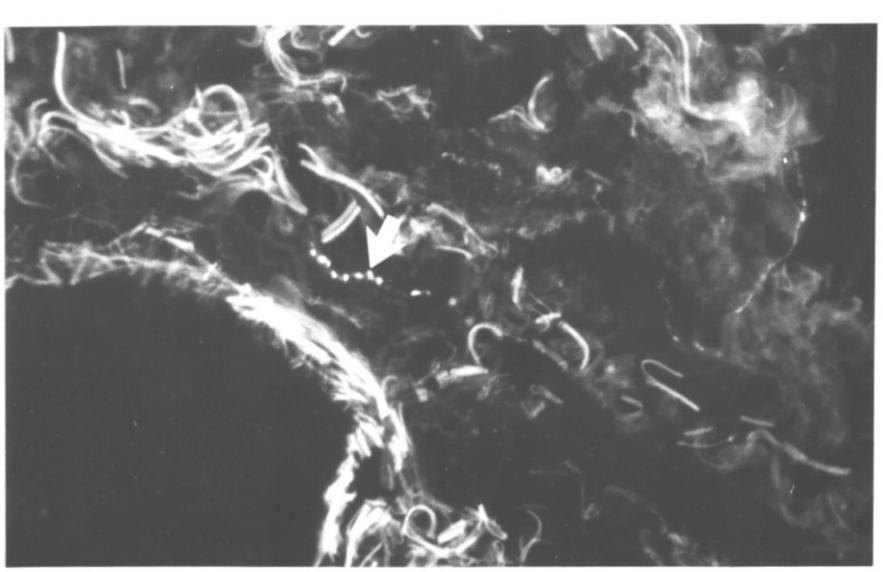


Fig.23 Adrenergic nerve in subsegmental airway x400

PLATE 8: Immunogold/Silver enhancement and indirect immunofluorescence

Fig.24 Tyrosine hydroxylase in BSM and ganglion x800

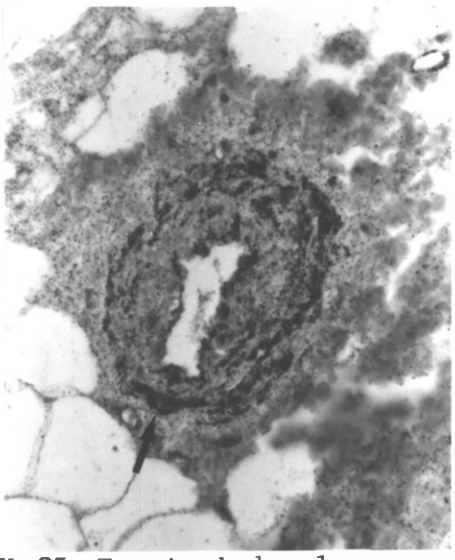


Fig.25 Tyrosine hydroxylase around arteriole x1000

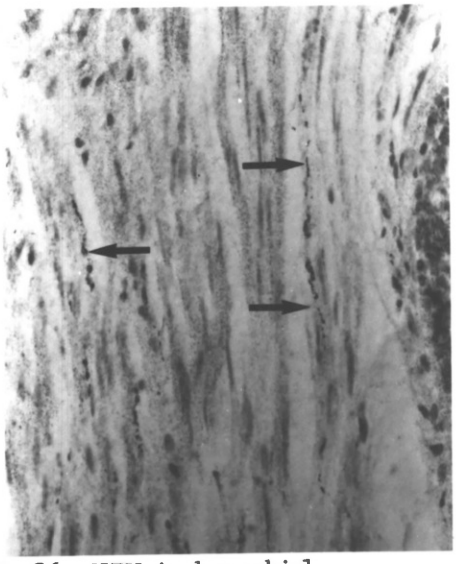


Fig.26 NPY in bronchial smooth muscle x800

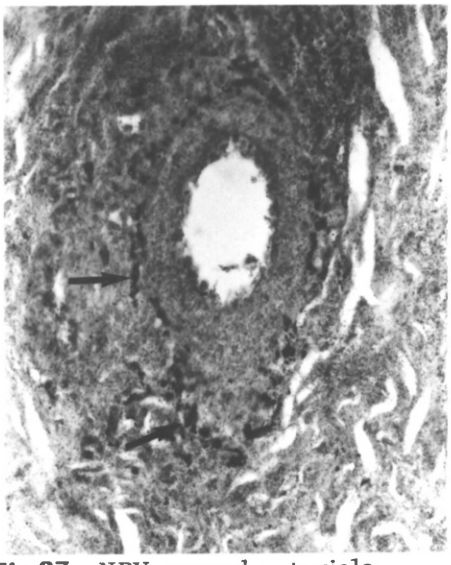


Fig.27 NPY around arteriole x1000

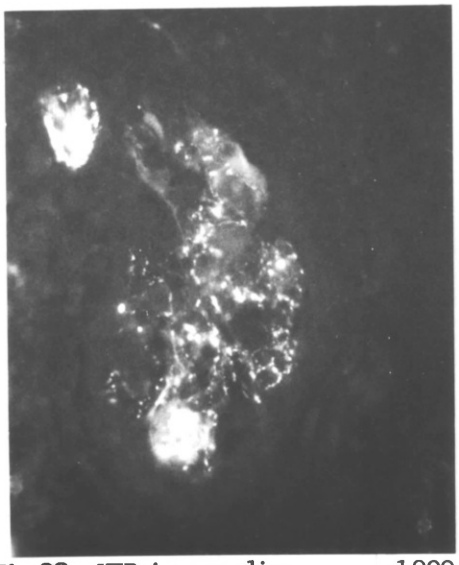


Fig.28 VIP in ganglion x1000

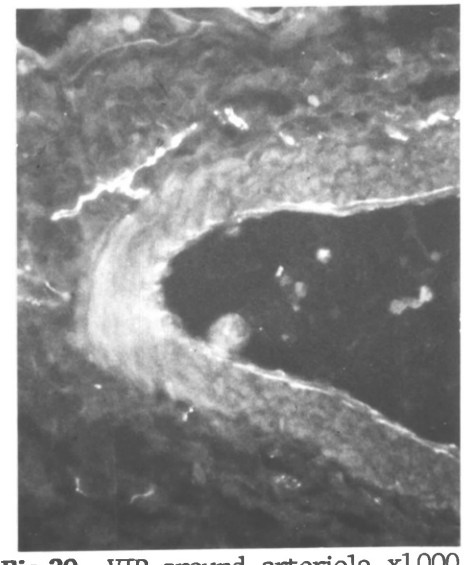


Fig.29 VIP around arteriole x1000

3.2. QUANTITATIVE ASSESSMENTS: NORMAL AND DISEASED LUNG.

This section contains the results of the quantitative assessments of the area on each section occupied by each effector structure of interest, the number of nerve fibres per mm length of airway epithelium and the quantification of the distribution of substance P and CGRP. The assessments were carried out on samples of lung tissue from 'normal' lung, tissue from the group of 'young' patients, and tissue from patients with bronchiectasis, cystic fibrosis and asthma.

3.2.1. Relative size of effector structures: point counts.

Point counts were made on sections of formalin-fixed, paraffin embedded tissue that had been stained by the immunogold technique for neuropeptides. The aim was to determine the relative area of the section occupied by each effector structure such that the determinations of the density of nerves could be more readily interpreted.

TABLE 6.

Mean (±SEM) percentage areas of sections occupied by each effector structure

Group	Gland	BSM	Ganglia	LP	EP	BV'S
'Normal'	20.3±3.0	9.0±2.6	2.0±0.4	15.5±3.1	4.6±1.1	6.2±1.1
'Young'	21.6±3.3	5.1±1.1	1.9±0.5	12.3±1.7	3.4±0.9	6.0±0.4
CF	30.1±3.6	7.9±1.4	2.9±0.6	9.3±1.5	3.3±0.5	8.6±0.7
B/E	25.3±2.6	7.0±2.5	2.9±0.6	21.2±3.6	6.8±1.9	6.1±0.4
Asthma	21.5±2.0	11.4±2.8	2.4±0.4	17.6±0.4	3.0±0.2	7.6±1.0

Results of comparison of the data using the Mann Whitney U test.

a Submucosal gland - there were no significant differences between any of the groups.

b. Bronchial smooth muscle (BSM) - The asthmatic tissue had a significantly greater amount of smooth muscle than the young tissue ($p<0.05$) otherwise there were no differences between the groups.

c. Ganglia - there were no significant differences between any of the groups in the area of the tissue sections occupied by ganglia.

d. Lamina propria (LP) - Lamina propria varied between sections and between groups. A significantly greater ($p<0.05$) proportion of the section was occupied by the lamina propria of the bronchiectatic and asthmatic tissue when compared to the 'young' or cystic fibrosis tissue but there was no difference between any of the groups and the normal tissue.

e. Epithelium (EP) - There were no significant differences in the area of the section occupied by epithelium between any of the groups.

f. Blood vessels (BV'S) - CF tissue showed a significantly greater ($p<0.05$) proportion of the tissue occupied by blood vessels than tissue from 'young' or bronchiectatic patients. There were no differences between the normal tissue and any of the other groups.

3.2.2. Quantification of epithelial nerves.

The number of nerve fibres per mm length of epithelium was assessed in sections of formalin-fixed, paraffin-embedded human intrapulmonary airway. The sections were stained for PGP 9.5 using the immunogold silver enhancement technique. The samples were obtained from the patients listed in the following groups:-

TABLE 7.

Tissue used in the quantification of epithelial nerves.

Normal	Young	CF	B/E	Asthma
336	218	334	415	2
180	216	316	740	13
184	217	322	2330	19/84
189	277	327	542	28/77
205	333	321	236	256/86
206				
204				
n=7	n=5	n=5	n=5	n=5

TABLE 8.

Total epithelial length (mm) measured in each group:-

Normal	Young	CF	B/E	Asthma
220.2	230.9	133.0	138.4	119.0

TABLE 9.

Mean (±SEM) number of nerves per mm length of epithelium in each group.

Normal	Young	CF	B/E	Asthma
1.58±0.17	1.66±0.25	1.02±0.11	1.30±0.24	1.58±0.33

Comparison of the data using the Mann-Whitney U test. Application of the Mann-Whitney U test to the data revealed no significant difference in the numbers of nerve fibres per mm epithelial length when comparing normal tissue with tissue from the 'young', bronchiectasis and asthma groups. However, tissue from patients with cystic fibrosis showed a significantly lower number of nerve fibres ($p<0.05$). Comparison of CF with 'young' tissue also revealed a significantly lower mean number of nerve fibres per mm ($p<0.05$) but there was no difference in comparison with the asthmatic or bronchiectatic tissue.

3.2.3. Quantification of substance P and CGRP fibres.

Applying the immunogold/silver enhancement technique to paraffin sections of formalin-fixed human lung tissue, the distribution of SP and CGRP-immunoreactivity was studied in the patients listed in the following groups:-

TABLE 10.

Tissue used in the quantification of substance P and CGRP immunoreactivity.

Normal	Young	CF	B/E	Asthma
204	218	322	740	5/87
184	216	327	542	28/77
180	229	316	415	10
206	287	334	2330	19/84
336	215	321	236	13
189	217			
205	333			
n=7	n=7	n=5	n=5	n=5

3.2.3.1. Substance P.

TABLE 11.

Mean (±SEM) number of substance P fibres per section.

Group	Bronchial muscle	Ganglia	Lamina propria	Epithelium	Blood vessels	Number of sections
Normal	1.8±0.9	3.3±0.7	6.6±4.0	2.3±1.6	0.9±0.5	21
Young	0.1±0.01	2.4±0.4	1.4±0.4	0.6±0.3	0.6±0.3	21
CF	1.0±0.2	2.2±0.4	2.9±1.0	0.2±0.1	1.1±0.4	15
B/E	0	1.3±0.4	0.5±0.2	0	0.1±0.1	15
Asthma	0.2±0.2	3.6±1.7	7.0±6.3	0.5±0.3	0.5±0.3	15

(Submucosal gland was negative for SP.)

Results of the comparison of the groups using the Kolmogorov-Smirnov 2-sample test. The areas compared were the ganglia, lamina propria and the epithelium. Blood vessels and bronchial smooth muscle were so infrequently innervated that it was not considered relevant to do a comparative test.

a. Ganglia - there were no significant differences between any of the groups except between the bronchiectatic tissue, which had the lowest mean score, and the asthmatic tissue which scored the highest ($p<0.05$).

b. Lamina propria - The normal tissue scored significantly higher than all the other groups except asthma ($p<0.05$). However, both normal and asthmatic groups contained samples which stained abnormally highly for substance P which could account for the observed differences.

c. Epithelium - there were no significant differences observed between the groups, except for the bronchiectatic tissue in which no substance P fibres were observed at any time in the epithelium.

3.2.3.2. CGRP.

TABLE 12

Mean (±SEM) number of CGRP fibres per section.

Group	Bronchial muscle	Ganglia	Lamina propria	Epithelium	No. sections.
Normal	0.9±0.4	6.1±0.9	8.4±2.6	0.8±0.5	21
Young	0.1±0.01	2.8±0.1	2.8±1.1	0.4±0.3	21
CF	0.2±0.1	3.0±0.9	3.7±0.8	0.5±0.2	15
B/E	0	1.2±0.4	0.8±0.4	0.3±0.2	15

(Submucosal gland and blood vessels were negative on tissue sections used for quantification).

Results of Kolmogorov-Smirnov 2-sample test. The counts for bronchial smooth muscle were not included due to the infrequent occurance of any nerve fibres.

a. Ganglia - There were no significant differences between the scores for any of the groups except for that between normal and bronchiectatic tissue which provided the highest and lowest scores respectively ($p<0.05$).

b. Lamina propria - The brochiectatic tissue had a significantly lower score than tissue from either the young or normal groups ($p<0.05$ and $p<0.01$ respectively). Otherwise there were no significant differences between the groups.

c. Epithelium - All the scores were significantly different ($p<0.001$) when compared with one another. The normal tissue had the highest score followed by the CF tissue, then the 'young' tissue and finally the bronchiectatic tissue. The statistically significant differences are partly attributable to the infrequency of CGRP nerves in the epithelium (Neuroendocrine cells were not included in the score).

3.3. SEMI-QUANTITATIVE ASSESSMENTS: NORMAL AND DISEASED LUNG.

The aim of the semi-quantitative assessments was to determine the relative density and distribution of neural markers that occured frequently (such that counts of individual nerve profiles was impractical). These markers included cholinesterase histochemistry for cholinergic nerves, catecholamine fluorescence for 'aminergic' nerves and immunoreactivity for VIP, tyrosine hydroxylase and NPY. The relative densities of the markers were assessed on a scale of 0-5, where a score of 0 indicated a negative section and a score of 5, the maximum density of innervation observed in any section. The appropriate analysis of variance (see Methods, section 2.6) was applied to the semi-quantitative assessment to determine the variability between normal and disease conditions, between airway levels and between individual patients (within groups) for each type of neural marker. The residual component incorperates the variation between samples from the same patient.

The results of the analysis of variance are presented in table form where SS = sums of squares of the semi-quantitative data and MS = mean squares. The F value is applied to a table of its probability distribution using the appropriate degrees of freedom to obtain the p value. A table of the mean scores $\pm$ the standard error of the mean (SEM) for each type of neural marker in each effector structure is also given for each group.

3.3.1. Semi-quantitative assessment of the density of cholinergic nerves.

The distribution of cholinergic nerves as revealed by cholinesterase histochemistry was quantified in tissue from 5 normal patients, 5 patients with cystic fibrosis, 2 bronchiectatic patients and 1 asthmatic patient. The cases used are listed in the following table:-

TABLE 13.

Tissue used in the semi-quantitative assessment of the density of cholinergic nerves.

Normal	CF	BE	Asthma
149	211	500	313
155	222	340	
152	283		
154	175		
156	172		
n=5	n=5	n=2	n=1

3.3.1.1. Results of the 2-way analysis of variance.

Semi-quantitative assessment was applied to the normal and cystic fibrosis tissue: insufficient tissue was available from patients with either bronchiectasis or asthma. Subjectively, the bronchiectatic tissue showed a reduction in the density of stain, but there was no obvious difference between the asthmatic tissue and the normal tissue.

Two sections from airway levels I-IV from each case were assessed: the results of the hierarchical analysis of variance are presented in table 16. (Table 14 gives the mean score for each group $\pm$ the standard error of the mean, SEM)

TABLE 14.

Mean scores (±SEM) of the density of cholinergic nerves in **normal** and **CF** tissue.

Structure	**Normal**	**CF**
Submucosal gland	4.10±0.1	4.10±0.1
Bronchial smooth muscle	4.70±0.1	4.70±0.1
Lamina propria	3.40±0.1	3.50±0.1
Pulmonary vessels	0.33±0.1	0.40±0.1
Bronchial vessels	0.83±0.12	0.84±0.1

TABLE 15.

Mean scores (±SEM) of the density of cholinergic nerves at different **airway levels** in normal lung.

Structure	**Airway level**				
	I	**II**	**III**	**IV**	**p**
Submucosal gland	4.4±0.2	4.0±0.2	3.9±0.1	/	n.s.
Bronchial smooth muscle	4.9±0.1	4.5±0.2	4.0±0.1	3.5±0.2	<0.01
Lamina propria	3.8±0.4	3.5±0.2	3.5±0.2	3.3±0.2	<0.05
Bronchial vessels	1.2±0.4	1.0±0.2	0.2±0.1	/	<0.01
Pulmonary vessels	0.4±0.2	0.3±0.2	/	/	<0.05

TABLE 16.

Cholinergic nerves: Hierarchical analysis of variance of the density in **normal** and **CF** airway.

Structure	**Variation Between**	**SS**	**Degrees of freedom**	**MS**	**F**	**p**
Submucosal gland (levels I, II & III)	Conditions	0.013	1	0.013	0.054	n.s
	Patients	4.003	9	0.445	1.854	n.s
	Levels	11.67	29	0.402	1.680	n.s
	Residual	4.8	20	0.240		
Bronchial smooth muscle	Conditions	0.015	1	0.015	0.128	n.s
	Patients	2.80	9	0.311	2.66	$p<0.05$
	Levels	10.87	39	0.279	2.39	$p<0.05$
	Residual	3.5	30	0.117		
Lamina propria	Conditions	0.2	1	0.2	0.61	n.s
	Patients	8.6	9	0.95	2.87	$p<0.05$
	Levels	29.0	39	0.74	2.22	$p<0.05$
	Residual	10.0	30	0.33		
Pulmonary vessels	Conditions	0.105	1	0.105	0.7	n.s
	Patients	2.245	9	0.25	1.667	n.s
	Levels	17.625	39	0.452	3.013	$p<0.01$
	Residual	4.5	30	0.15		
Bronchial vessels	Conditions	0.015	1	0.015	0.069	n.s.
	Patients	5.500	9	0.611	2.861	$p<0.05$
	Level	56.875	39	1.457	6.714	$p<0.001$
	Residual	6.5	30	0.217		

3.3.1.2. Summary of Cholinergic data.

Qualitative assessment of the available tissue showed no apparent differences in the density of cholinergic innervation between samples from normal patients and samples from patients with cystic fibrosis or asthma. A slight reduction in the density of innervation in the bronchiectatic tissue was observed. Semi-quantitative assessment of the density of innervation showed no significant differences between normal and cystic fibrosis tissue when the data was subjected to a hierarchical analysis of variance. In general, the density of innervation decreased with increasing airway generations. This was reflected in the analysis of variance as a significant difference between airway levels. This was true of all structures except the submucosal glands which showed no differences in the density of innervation with increasing airway generation. The reduction in density of innervation with increasing airway generation was particularly marked in relation to bronchial and pulmonary vessels: the analysis of variance showed a highly significant difference between airway levels for both types of vessel ($p<0.001$ and $p<0.01$ respectively). However, there were significant differences in the bronchial vascular innervation between individual patients ($p<0.05$) which may account for the observed differences.

3.3.2. Semi-quantitative assessment of the density of VIPergic innervation.

Antibodies to vasoactive intestinal peptide were applied to cryostat sections of benzoquinone fixed tissue from airway levels I-III and visualised by indirect immunofluorescence, and to paraffin sections of formalin-fixed intrapulmonary airway when they were visualised by the immunogold/silver-enhancement technique. Tissue was used from patients listed in the following groups:-

TABLE 17.

Benzoquinone-fixed tissue used in the assessment of the density of VIP-immunoreactivity.

Normal	CF	BE	Asthma
262	211	500	313
230	316	340	
236	322		
336	327		
332	321		
n=5	n=5	n=2	n=1

TABLE 18.

Formalin-fixed, paraffin-embedded tissue used in the assessment of the density of VIP immunoreactivity.

Normal	Young	CF	BE
204	215	316	540
336	217	327	2330
180	216	322	415
206	287	321	236
189	277	334	740
205	229	318	
184	333		
n=7	n=7	n=6	n=5

3.3.2.1. Analysis of variance; benzoquinone-fixed tissue.

Three sections from each sample were assessed semi-quantitatively at each of 3 airway levels. The results of the semi-quantitative assessment of the density of VIPergic immunofluorescence were subjected to a hierarchical analysis of variance:-

TABLE 19.

Mean scores (±SEM) of the density of VIP-immunoreactivity in benzoquinone-fixed **normal** and **CF** tissue.

Structure	**Normal**	**CF**
Submucosal gland	3.95±0.85	2.24±0.65
Bronchial smooth muscle	4.00±0.93	2.80±1.09

TABLE 20.

Mean scores (+SEM) of the density of VIP at diferent airway levels in **normal** lung.

Structure	**Airway level**				
	I	II	III	IV	p
Submucosal gland	3.67±0.23	4.07±0.15	4.20±0.20	/	<0.001
Bronchial smooth muscle	4.20±1.08	3.80±0.11	3.93±1.16	3.93±0.16	<0.001

TABLE 21.

VIPergic nerves: Hierarchical analysis of variance of the density in benzoquinone-fixed **normal** and **CF** tissue.

Structure	**Variation Between**	**SS**	**Degrees of freedom**	**MS**	**F**	**p**
Submucosal gland	Conditions	65.5	1	65.5	399.39	$p<0.001$
	Patients	20.39	8	2.54	15.49	$p<0.001$
	Levels	10.89	23	0.743	2.89	$p<0.001$
	Residual	9.34	57	0.164		
Bronchial smooth muscle	Conditions	27.78	1	27.78	118.72	$p<0.001$
	Patients	28.21	8	3.53	15.09	$p<0.001$
	Levels	57.17	23	2.49	10.64	$p<0.001$
	Residual	13.33	57	0.234		

There was a reduction in density of VIPergic nerves in both the submucosal gland and bronchial smooth muscle of both cases of bronchiectasis. No difference was observed in the density of innervation in the tissue from the asthmatic patient.

3.3.2.2. Analysis of variance; formalin-fixed, paraffin-embedded tissue

The results of the semi-quantitative assessment of the density of VIPergic nerves in 2 sections from each sample were subjected to a 2-way analysis of variance the results of which are presented in the following tables:-

TABLE 22.

Mean scores (±SEM) of the density of VIP-immunoreactivity in formalin-fixed and paraffin-embedded tissue.

Structure	Normal	CF	BE	Young
Submucosal gland	3.64±0.33	1.90±0.25	0.44±0.17	2.80±0.26
Bronchial smooth muscle	4.09±0.33	2.70±0.36	0.80±0.34	2.90±0.32
Ganglia	3.36±0.29	1.09±0.16	0.40±0.19	2.40±0.42
Lamina propria	1.82±0.37	1.36±0.15	0.70±0.23	1.14±0.29
Pulmonary vessels	1.00±0.12	1.09±0.26	0.40±0.17	0.57±0.21
Bronchial vessels	1.18±0.24	0.56±0.26	0.40±0.17	0.64±0.24

TABLE 23.

VIPergic nerves: 2-way analysis of variance of the density in **normal** and **CF** tissue.

Structure	**Variation Between**	**SS**	**Degrees of freedom**	**MS**	**F**	**p**
Submucosal gland	Conditions	18.41	1	18.41	37.28	p<0.001
	Within gp	8.05	9	0.894	1.81	n.s.
	Residual	4.45	9	0.494		
Bronchial smooth muscle	Conditions	9.8	1	9.8	5.128	p<0.05
	Within gp	13.8	9	0.422	0.221	n.s.
	Residual	10.818	9	1.911		
Ganglia	Conditions	26.182	1	26.182	26.662	p<0.001
	Within gp	2.364	10	0.236	0.240	n.s.
	Residual	9.818	10	0.982		
Lamina propria	Conditions	2.45	1	2.45	4.367	n.s.
	Within gp	13.45	9	1.494	2.663	n.s.
	Residual	5.05	9	0.561		
Pulmonary vessels	Conditions	1.136	1	1.136	2.119	n.s.
	Within gp	5.364	10	0.536	1.0	n.s.
	Residual	5.364	10	0.536		
Bronchial vessels	Conditions	0.455	1	0.046	0.061	n.s.
	Within gp	5.091	10	0.509	0.683	n.s.
	Residual	7.455	10	0.746		

TABLE 24.

VIPergic nerves: 2-way analysis of variance of the density in **normal** and **bronchiectatic** tissue.

Structure	Variation Between	SS	Degrees of freedom	MS	F	p
Submucosal gland	Conditions	57.8	1	57.80	123.85	p<0.001
	Within gp	5.8	9	0.644	1.38	n.s.
	Residual	4.2	9	0.467		
Bronchial smooth muscle	Conditions	54.45	1	54.45	37.552	p<0.001
	Within gp	3.45	9	0.383	0.264	n.s.
	Residual	13.05	9	1.45		
Ganglia	Conditions	54.45	1	54.45	160.670	p<0.001
	Within gp	5.05	9	0.561	1.655	n.s.
	Residual	3.05	9	0.339		
Lamina propria	Conditions	12.8	1	12.8	11.294	p<0.01
	Within gp	4.8	9	0.533	0.747	n.s.
	Residual	10.2	9	1.133		
Pulmonary vessels	Conditions	2.45	1	2.45	20.99	p<0.01
	Within gp	4.25	9	0.472	4.046	p<0.05
	Residual	1.05	9	0.117		
Bronchial vessels	Conditions	4.05	1	4.05	8.198	p<0.05
	Within gp	2.05	9	0.228	0.462	n.s.
	Residual	4.45	9	0.494		

TABLE 25.

VIPergic nerves: 2-way analysis of variance of the density in **normal** and **'young'** tissue.

Structure	Variation Between	SS	Degrees of freedom	MS	F	p
Submucosal gland	Conditions	5.0	1	5.0	11.26	p<0.01
	Within gp	9.5	9	1.022	2.302	n.s.
	Residual	4.0	9	0.444		
Bronchial smooth muscle	Conditions	4.323	1	4.323	2.231	n.s.
	Within gp	14.607	13	1.124	0.580	n.s.
	Residual	25.179	13	1.937		
Ganglia	Conditions	8.036	1	8.036	7.759	p<0.05
	Within gp	23.464	13	1.805	1.743	n.s.
	Residual	13.464	13	1.036		
Lamina propria	Conditions	0.893	1	0.893	0.998	n.s.
	Within gp	20.179	13	1.552	1.734	n.s.
	Residual	11.607	13	0.895		
Pulmonary vessels	Conditions	0.143	1	0.143	0.65	n.s.
	Within gp	10.714	13	0.824	3.75	p<0.05
	Residual	2.857	13	0.220		
Bronchial vessels	Conditions	1.286	1	1.286	3.546	n.s.
	Within gp	11.429	13	0.879	2.424	n.s.
	Residual	4.714	13	0.363		

TABLE 26.

VIPergic nerves: 2-way analysis of variance of the density in **CF** and **'young'** tissue.

Structure	**Variation Between**	**SS**	**Degrees of Freedom**	**MS**	**F**	**p**
Submucosal gland	Conditions	4.05	1	4.05	8.198	$p<0.05$
	Within gp	6.05	9	0.672	1.360	n.s.
	Residual	4.45	9	0.494		
Bronchial smooth muscle	Conditions	1.25	1	1.25	0.918	n.s.
	Within gp	17.45	9	1.939	1.425	n.s.
	Residual	12.25	9	1.361		
Ganglia	Conditions	7.2	1	7.2	4.38	n.s.
	Within gp	3.2	9	0.356	0.217	n.s.
	Residual	14.8	9	1.644		

3.3.2.3. Correlation of the score for VIP in submucosal gland with the precentage area of gland.

The raw data indicated that there might be a correlation between the score for VIP in the submucosal gland and the area of the section occupied by the gland. This hypothesis was tested in the following way. The percentage area of the tissue section occupied by submucosal gland was assessed using a point counting method (see Methods, section 5.0, Results, section 2.1) and this was correlated with VIP score using Spearman's correlation coefficient. The results are presented in tables 27 and 28 and are illustrated by graphs in figure 3.1, a-d.:-

TABLE 27.

% area of submucosal gland per section and corresponding score for VIP.

Group	% Area of Gland per section.	Score for VIP.
'Normal'	13.8	4
	13.0	5
	35.3	1
	22.9	3
	28.9	2
	22.9	4
'Young'	23.3	3
	23.3	4
	22.7	4
	28.6	2
	32.7	1
	25.0	3
CF	39.2	1
	36.1	2
	19.3	3
	30.0	2
	25.8	3
	28.0	2
Bronchiectais	34.2	0
	20.3	1
	27.3	0
	24.6	1
	20.0	2
	22.5	1

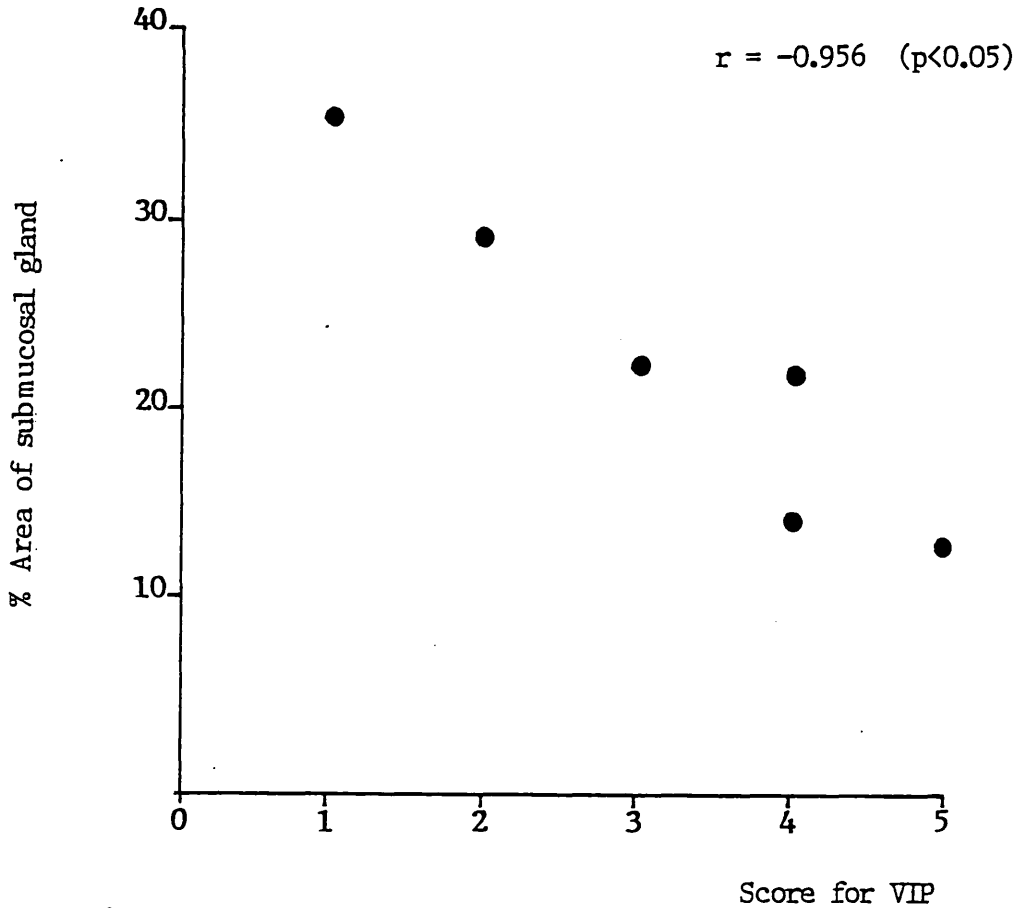
TABLE 28.

Spearman's rank correlation coefficient for the % area of submucosal gland and the score for VIP.

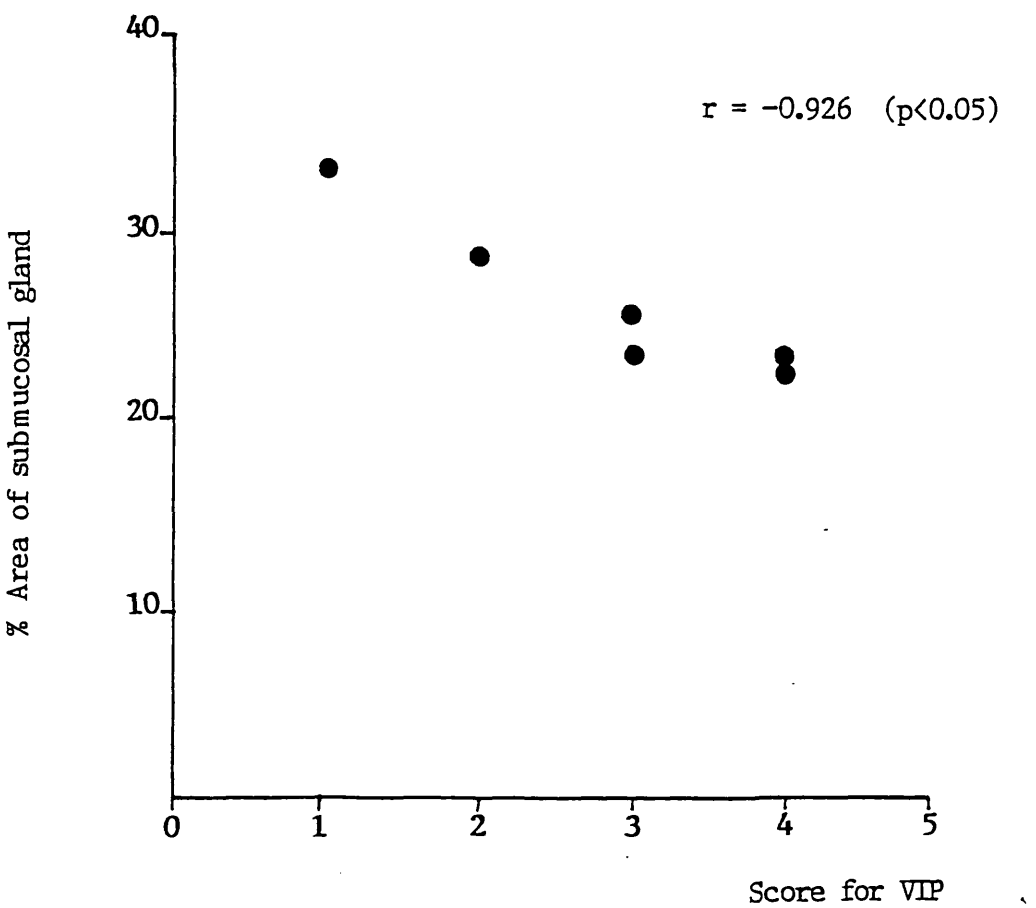
Group	Correlation coefficient	n	p
Normal	-0.956	6	$p<0.05$
CF	-0.926	6	$p<0.05$
Young	-0.926	6	$p<0.05$
BE	-0.926	6	$p<0.05$

Fig. 3.1 Relationship between the % area of submucosal gland per section and the score for VIP in different patient groups.

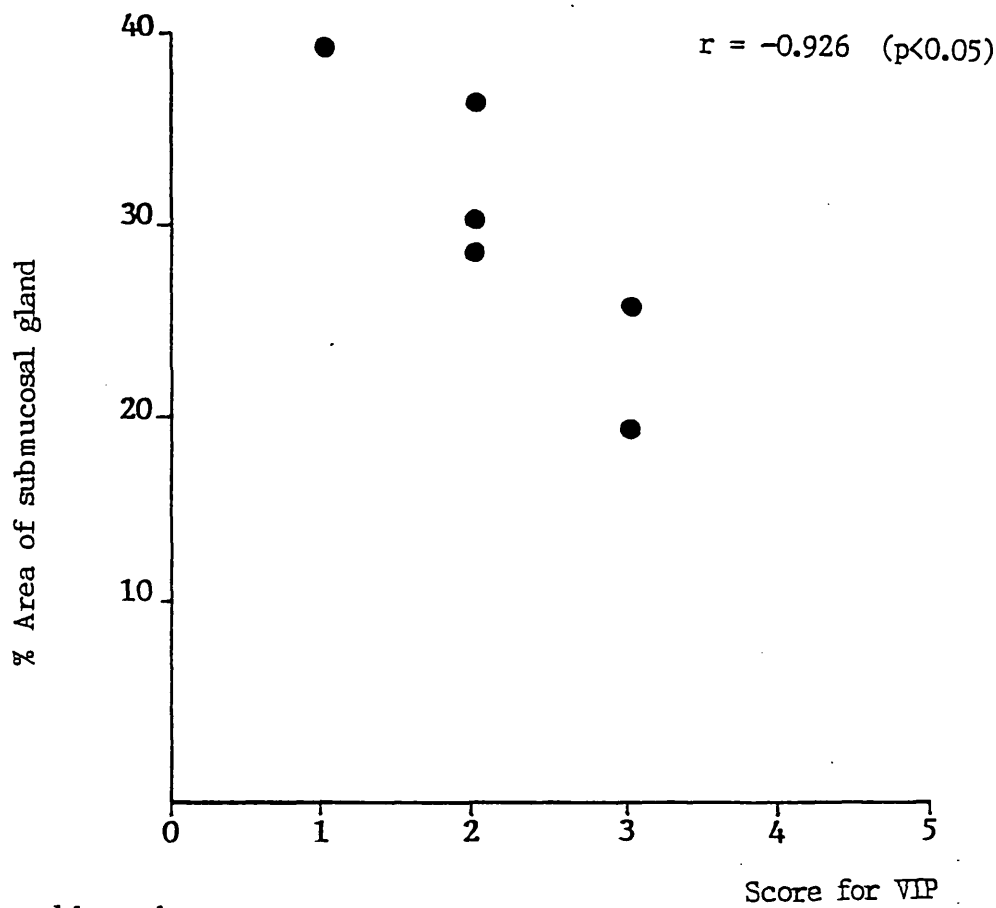
a) 'Normal'



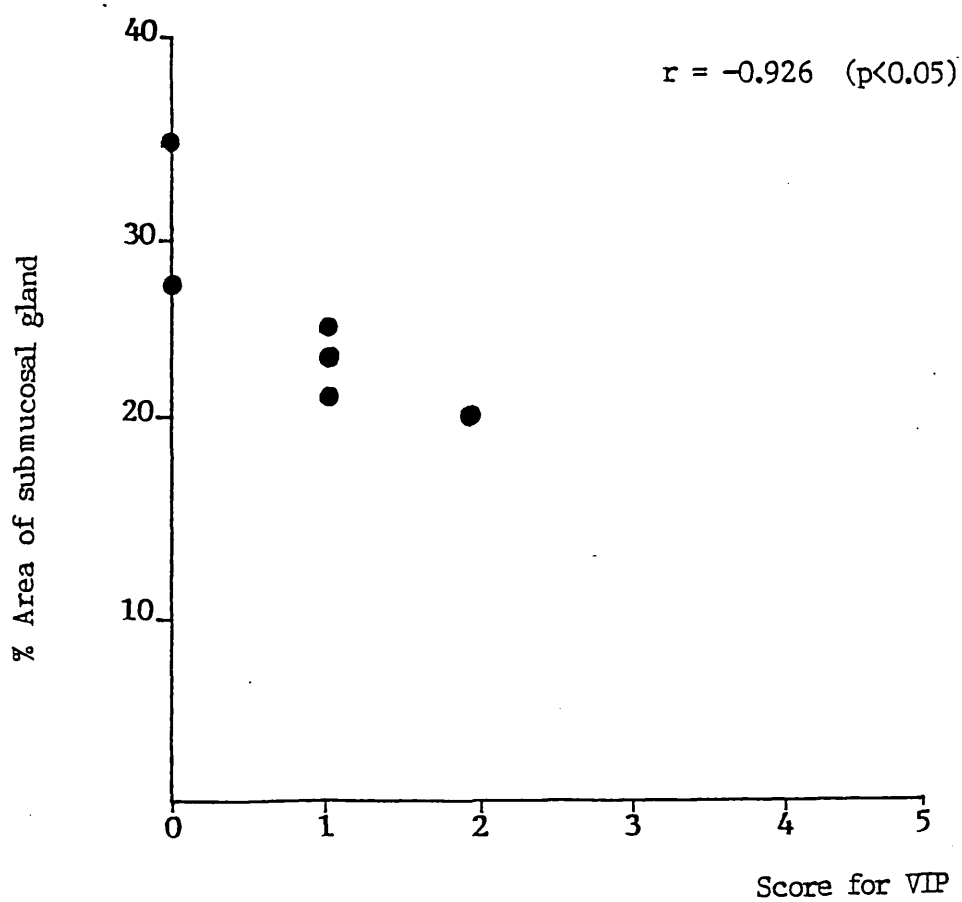
b) 'Young'



c) Cystic fibrosis



d) Bronchiectasis



3.3.2.4. Summary of VIP data.

Comparison of the density of VIP immunoreactivity in benzoquinone fixed samples of human airway revealed significant differences between airway levels, between patients within the same group and between normal tissue and tissue taken from patients with CF. The hierarchical analysis of variance indicated a high variability depending on where the sample was taken from in terms of both the airway level and the individual patient. However, this variablity in the samples was insufficient to account for a marked decrease in the density of VIP in CF samples compared to the 'normal' tissue ($p<0.001$). This decrease was particularly marked in the submucosal gland (see Plate 3, figs. 10 and 11) but was also present in the bronchial smooth muscle. A qualitative assessment of the two cases of bronchiectasis also revealed a marked reduction in the density of VIP nerves. The density of VIP-immunoreactivity in the one available sample of asthmatic airway did not appear to be any different from that observed in the normal tissue.

Two-way analysis of variance of the semi-quantitative assessment of VIPergic innervation in formalin fixed paraffin-embedded samples of intrapulmonary airways indicated differences between normal and CF tissue. Again the difference was most marked in the submucosal gland ($p<0.001$) and was present to a lesser extent in the bronchial smooth muscle ($p<0.05$). Analysis of the scoring for other areas of the section revealed reduced staining in the ganglia in CF ($p<0.001$), but no marked reduction of stain in the lamina propria or blood vessels.

Bronchiectatic tissue showed a significant reduction in the density of VIP immunoreactivity in all components of the airways; the reduction was most marked in the submucosal gland ($p<0.001$).

Tissue from the 'young' group showed a reduction in the scores for submucosal gland ($p<0.01$) and ganglia ($p<0.05$) but no difference in the other airway components.

A comparison was made between the CF scores and those for the 'young' group as an alternative contol to the normal group (the 'young' group being of similar mean age to the CF group). This revealed that the CF tissue scored significantly lower than the tissue from the 'young' group for VIP in the submucosal gland ($p<0.05$) but there was no difference in the bronchial smooth muscle or ganglia.

Calculation of the Spearman's rank correlation coefficient for gland and the corresponding score for VIP indicated a strong negative correlation between the two i.e. the sections that had the highest percentage of submucosal gland had the lowest scores for VIP.

3.3.3. Semi-quantitative assessment of the density of aminergic nerves.

The distribution of adrenergic nerves as revealed by glyoxic acid-induced fluorescence of catecholamines, was examined in 'normal' airways from 23 patients and in airways from 7 patients with CF, 2 bronchiectatic (BE) patients and 1 asthmatic patient. However quantification of the aminergic nerves using this technique was only possible in 'normal' airways from 7 patients. Tissue availability from patients with cystic fibrosis was infrequent and thus prevented simultaneous comparison of several cases at one time: by the time tissue from a few cases had been collected, the catecholamine content of the tissue collected earlier had deteriorated. Those cases which were assessed qualitatively are listed in table 26 below.

TABLE 29.

Tissue used in the assessment of the density of aminergic nerves.

Normal*	Normal	CF	BE	Asthma
149	136	327	500	313
152	40	222	340	
155	77	211		
154	85	321		
186	86	358		
167	87	334		
168	122	316		
	80			
	230			
	262			
	332			
	336			
	236			
	302			
	365			
	353			
n=7	n=16	n=7	n=2	n=1

*These cases were both qualitatively and quantitatively assessed. There was no apparent qualitative difference in the distibution or density of nerves in CF or asthma. There was however a marked reduction in the density of nerves observed in the two cases of bronchiectasis.

3.3.3.1. Results of the 2-way analysis of variance.

The results of the semi-quantitative analysis of the distribution of adrenergic nerves in normal airways are presented below. Two sections from each sample at three airway levels (I-III) from each case were assessed and results are presented in a hierarchical analysis of variance.

TABLE 30.

Mean scores (±SEM) of the density of aminergic nerves in **'normal'** lung.

Structure	**Airway level**				
	I	**II**	**III**	**IV**	**p**
Submucosal gland	2.93±0.25	3.14±0.18	3.0±0.21	/	n.s.
Bronchial smooth muscle	0.5±0.20	0.79±0.21	0.86±0.66	0.5±0.10	n.s.
Ganglia	0.86±0.31	1.5±0.34	0.71±0.13	/	<0.01
Pulmonary vessels	0.85±0.23	0.43±0.14	0.57±0.14	/	n.s.
Bronchial vessels	2.29±0.19	2.14±0.23	1.78±0.32	1.8±0.14	n.s.

TABLE 31.

Aminergic nerves: Hierarchical analysis of variance of the density in normal lung.

Structure	Variation Between	SS	Degrees of freedom	MS	F	p
Submucosal gland	Patients	20.81	6	3.468	19.37	p<0.05
	Levels	0.462	2	0.231	1.291	n.s
	Samples	4.9	5	0.98	5.475	p<0.01
	Residual	5.0	28	0.179		
Bronchial smooth muscle	Patients	7.572	6	1.262	8.825	n.s.
	Levels	0.619	2	0.310	2.164	n.s.
	Samples	7.711	5	1.542	10.783	p<0.001
	Residual	4.0	28	0.143		
Ganglia	Patients	22.951	6	3.825	26.767	p<0.05
	Levels	7.910	2	3.595	21.666	p<0.01
	Samples	15.481	5	3.096	21.666	p<0.001
	Residual	4.0	28	0.1429		
Pulmonary vessels	Patients	7.905	6	1.318	14.803	n.s.
	Levels	0.191	2	0.096	1.079	n.s
	Samples	3.809	5	0.762	8.562	p<0.001
	Residual	2.50	28	0.089		
Bronchial vessels	Patients	16.29	6	2.715	11.703	n.s.
	Levels	1.004	2	0.502	2.164	n.s
	Samples	15.0	5	3.0	12.931	p<0.001
	Residual	6.5	28	0.232		

3.3.3.2. Summary of aminergic data.

Qualitatively, there was no change in cystic fibrosis or asthma. However, there was a marked reduction in the density of adrenergic nerves in bronchiectasis but this difference could not be tested statistically due to the small sample size (n=2).

The hierarchical analysis of variance of the semi-quantitative assessment of the density of adrenergic nerves in 'normal' lung revealed a significant variation between airway levels in the adrenergic innervation of cholinergic ganglia. There was, however, a significant amount of variation between patients and between samples taken from individual patients, in all bronchial components. Therefore any findings of differences between airway levels cannot be assumed to be 'real' differences.

3.3.4. Semi-quantitative assessment of the density of tyrosine hydroxylase.

Antibodies to tyrosine hydroxylase were applied to formalin-fixed, paraffin embedded intrapulmonary airway and visualised using the immunogold silver-enhancement technique in retrograde studies on cystic fibrosis, bronchiectatic and asthmatic tissue. Two groups of tissue were used as control tissue: 'Normal' airways from lungs resected for carcinoma and 'young' which comprised tissue taken from patients with an average age of 28 and who suffered from diseases other than carcinoma of the lung such as bronchitis and congenital emphysema.

Antibodies to tyrosine hydroxylase, however, failed to stain the asthmatic tissue. Therefore data are presented only for the other groups.

TABLE 32.

Tissue used in the assessment of the density of tyrosine hydroxylase-immunoreactivity.

Normal	Young	CF	BE
204	277	316	740
336	217	327	540
206	229	322	2330
189	215	321	415
205	287	334	
180	333		
184			
n=7	n=6	n=5	n=4

3.3.4.1. Results of the 2-way analysis of variance.

Two sections of each case were assessed on a semi-quantitative basis and the results are expressed as a two-way analysis of variance.

TABLE 33.

Mean scores (± SEM) of the density of tyrosine hydroxylase-immunoreactivity.

Structure	Normal	CF	BE	Young
Submucosal gland	3.33±0.15	3.00±0.30	1.75±0.17	2.40±0.20
Bronchial smooth muscle	1.33±0.15	1.10±0.19	0.50±0.20	1.25±0.14
Ganglia	4.08±0.15	3.60±0.28	2.75±0.27	3.75±0.19
Pulmonary vessels	4.17±0.22	3.50±0.32	2.75±0.17	3.50±0.30
Bronchial vessels	4.33±0.15	4.20±0.21	3.38±0.28	4.42±0.16

TABLE 34.

Tyrosine hydroxylase: 2-way analysis of variance of the density in **'normal'** and **CF** tissue.

Structure	**Variation Between**	**SS**	**Degrees of freedom**	**MS**	**F**	**p**
Submucosal gland	Conditions	0.45	1	0.45	3.856	n.s.
	Within gp	6.25	9	0.694	5.950	$p<0.01$
	Residual	1.05	9	0.117		
Bronchial smooth muscle	Conditions	0.45	1	0.45	1.975	n.s.
	Within gp	3.25	9	0.361	1.585	n.s.
	Residual	2.05	9	0.228		
Ganglia	Conditions	1.25	1	1.25	1.216	n.s.
	Within gp	4.05	9	0.45	0.438	n.s.
	Residual	9.25	9	1.028		
Pulmonary vessels	Conditions	0.8	1	0.8	0.783	n.s.
	Within gp	4.2	9	0.467	0.457	n.s.
	Residual	9.2	9	1.022		
Bronchial vessels	Conditions	0.2	1	0.2	0.4737	n.s.
	Within gp	2.2	9	0.244	0.579	n.s.
	Residual	3.8	9	0.422		

TABLE 35.

Tyrosine hydroxylase: 2-way analysis of variance of the density in **'normal'** and **bronchiectatic** tissue.

Structure	**Variation Between**	**SS**	**Degrees of freedom**	**MS**	**F**	**p**
Submucosal gland	Conditions	9.0	1	9.0	20.99	$p<0.01$
	Within gp	0	7	0	0	n.s.
	Residual	3.0	7	0.429		
Bronchial smooth muscle	Conditions	2.25	1	2.25	9.0	$p<0.05$
	Within gp	1.75	7	0.25	1.0	n.s.
	Residual	1.75	7	0.25		
Ganglia	Conditions	4.0	1	4.0	14.001	$p<0.01$
	Within gp	5.75	7	0.821	2.875	n.s.
	Residual	2.0	7	0.286		
Pulmonary vessels	Conditions	6.25	1	6.25	25.0	$p<0.01$
	Within gp	3.75	7	0.536	2.143	n.s.
	Residual	1.75	7	0.25		
Bronchial vessels	Conditions	3.063	1	3.063	8.795	$p<0.05$
	Within gp	2.938	7	0.420	1.204	n.s.
	Residual	2.438	7	0.348		

TABLE 36.

Tyrosine hydroxylase: 2-way analysis of variance of the density in **'normal'** and **'young'** tissue.

Structure	Variation Between	SS	Degrees of freedom	MS	F	p
Submucosal gland	Conditions	4.167	1	4.167	11.956	p<0.01
	Within gp	5.33	11	0.485	1.391	n.s.
	Residual	3.83	11	0.349		
Bronchial smooth muscle	Conditions	0	1	0	0	n.s.
	Within gp	2.5	11	0.227	1.25	n.s.
	Residual	2.0	11	0.182		
Ganglia	Conditions	1.042	1	1.042	1.355	n.s.
	Within gp	6.458	11	0.587	0.763	n.s.
	Residual	8.458	11	0.769		
Pulmonary vessels	Conditions	1.5	1	1.5	3.367	n.s.
	Within gp	11.33	11	1.303	2.518	n.s.
	Residual	4.5	11	0.410		
Bronchial vessels	Conditions	0.042	1	0.042	0.315	n.s.
	Within gp	4.125	11	0.375	2.828	p<0.05
	Residual	1.458	11	0.133		

3.3.4.2. Summary of Tyrosine hydroxylase data.

There were no significant differences in the density of positive stain in any of the effector structures between samples taken from normal and cystic fibrosis patients.

Samples taken from patients with bronchiectasis scored consistently lower than samples taken from normal patients. This difference was reflected in the 2-way analysis of variance which showed significant differences in the scoring for tyrosine hydroxylase in all areas of interest between normal and bronchiectatic tissue. This difference could not be accounted for by the variation within samples from individual patients which showed no significant differences.

A reduction in density of stain was seen in the submucosal gland of samples taken from the 'young' group compared to the 'normal' samples. The analysis of variance showed the difference to be significant ($p<0.01$). There were no other significant differences between the 'normal' and 'young' samples. There was a significant variation ($p<0.05$) in the scoring for the bronchial vessels within samples from individual patients.

3.3.5. Semi-quantitative assessment of the density of neuropeptide Y.

Antibodies to NPY were applied to sections of formalin-fixed, paraffin-embedded intrapulmonary airway and visualised by the immunogold/silver enhancement technique. Tissue was obtained from patients in the following groups:-

TABLE 37.

Tissue used in the assessment of the density of NPY-immunoreactivity.

Normal	Young	CF	BE
204	333	322	415
336	218	316	740
206	229	334	542
189	277	327	2330
205	287	321	236
180	216		
184	217		
n=7	n=7	n=5	n=5

3.3.5.1. Results of the 2-way analysis of variance.

Two sections from each case were assessed on a semi-quantitative basis and the results are expressed as a two-way analysis of variance:-

TABLE 38.

Mean scores (±SEM) of the density of NPY immunoreactivity

Structure	Normal	CF	BE	Young
Bronchial smooth muscle	2.14±0.29	1.40±0.23	1.00±0.31	1.58±0.24
Ganglia	1.20±0.14	1.10±0.11	0.70±0.27	1.00±0.22
Pulmonary vessels	1.86±0.15	2.10±0.25	1.40±0.39	1.07±0.20
Bronchial vessels	1.64±0.21	1.40±0.17	1.00±0.35	0.93±0.17

TABLE 39.

NPY-immunoreactivity: 2-way analysis of variance of the density in **'normal'** and **CF** tissue.

Structure	**Variation Between**	**SS**	**Degrees of freedom**	**MS**	**F**	**p**
Bronchial	Conditions	5.0	1	5.0	22.052	p<0.01
smooth	Within gp	4.8	9	0.533	2.40	n.s
muscle	Residual	2.0	9	0.222		
Ganglia	Conditions	0.05	1	0.05	0.310	n.s
	Within gp	1.05	9	0.117	0.724	n.s
	Residual	1.45	9	0.161		
Pulmonary	Conditions	0	1	0	0	n.s
vessels	Within gp	1.8	9	0.2	0.45	n.s
	Residual	4.0	9	0.444		
Bronchial	Conditions	0.8	1	0.8	1.714	n.s
vessels	Within gp	1.8	9	0.2	0.429	n.s
	Residual	4.2	9	0.467		

TABLE 40.

NPY-immunoreactivity: 2-way analysis of variance of the density in **'normal'** and **bronchiectatic** tissue.

Structure	Variation Between	SS	Degrees of freedom	MS	F	p
Bronchial smooth muscle	Conditions	9.8	1	9.8	14.226	p<0.01
	Within gp	4.2	9	0.467	0.677	n.s
	Residual	6.2	9	0.689		
Ganglia	Conditions	1.25	1	1.25	2.647	n.s
	Within gp	3.45	9	0.383	0.812	n.s
	Residual	4.25	9	0.472		
Pulmonary vessels	Conditions	2.45	1	2.45	2.384	n.s
	Within gp	9.25	9	1.028	1.529	n.s
	Residual	6.05	9	0.672		
Bronchial vessels	Conditions	3.2	1	3.20	7.579	p<0.05
	Within gp	9.8	9	1.089	2.579	n.s
	Residual	3.8	9	3.80	0.422	

TABLE 41.

NPY-immunoreactivity: 2-way analysis of variance of the density in **'normal'** and **'young'** tissue.

Structure	Variation Between	SS	Degrees of freedom	MS	F	p
Bronchial smooth muscle	Conditions	4.321	1	4.321	4.590	n.s
	Within gp	12.750	13	0.981	1.047	n.s
	Residual	12.179	13	0.937		
Ganglia	Conditions	0.143	1	0.143	0.271	n.s
	Within gp	2.857	13	0.219	0.417	n.s.
	Residual	6.857	13	0.528		
Pulmonary vessels	Conditions	6.036	1	6.036	10.511	$p<0.01$
	Within gp	3.464	13	0.267	0.464	n.s.
	Residual	7.464	13	0.574		
Bronchial vessels	Conditions	3.571	1	3.571	6.915	$p<0.05$
	Within gp	6.714	13	0.517	1.237	n.s.
	Residual	5.429	13	0.418		

TABLE 42.

NPY-immunoreactivity: 2-way anaylsis of variance of the density in **CF** and **'young'** tissue.

Structure	Variation Between	SS	Degrees of freedom	MS	F	p
Bronchial	Conditions	0.45	1	0.45	0.447	n.s.
smooth	Within gp	1.45	9	0.161	0.160	n.s.
muscle	Residual	9.05	9	1.006		

3.3.5.2. Summary of NPY data.

There was a significant reduction in the scoring for NPY in the bronchial smooth muscle in cystic fibrosis compared with the 'normal' samples ($p<0.01$). There were no other differences in the NPY innervation between normal and cystic fibrosis tissue. Comparison of the CF tissue with samples taken from the 'young' group revealed no significant difference in the density of NPY stain in the bronchial smooth muscle. There was a reduction in density of NPY immunoreactivity in the bronchial smooth muscle of samples taken from patients with bronchiecatsis compared with samples taken from 'normal' lung ($p<0.01$), as well as a slight reduction in the innervation of bronchial vessels ($p<0.05$). There were no other significant differences. Samples taken from patients in the 'young' group did not show a reduction in density of stain in the bronchial smooth muscle compared to that seen in the samples of 'normal' airway. There was however a significant reduction in the density of stain associated with both bronchial and pulmonary vessels ($p<0.05$ and $p<0.01$ respectively) in the tissue from the young group compared to the normal tissue.

3.4.. COMPARATIVE RESULTS.

This section contains the results of the results of the radioimmunoassay of VIP and substance P in normal and CF lung and the determinations of serum cholinesterase activity in normal and asthmatic serum. These measurements were carried out for comparison with the results of the semi-quantitative assessments.

3.4.1. Results of Radioimmunoassay.

The results in the table below represent the mean of a duplicate measurement of the amount of VIP and substanceSP in picomoles per gramm of wet weight tissue.

3.4.1.1.. Amount of VIP.

TABLE 43.

The amount of VIP in samples of normal and CF lung.

Sample	Normal	Sample	Cystic fibrosis.
301	1.25	316	0.6
365	2.0	322	1.25
353	0.62	283	0.62
332	0.5	327	1.85
329	0.94	321	7.40
302	0.4	325	0.06
		358	1.50
mean score+ SEM	0.95±0.27		1.897±1.02

3.4.1.2.. Amount of substance P.

TABLE 44.

The amount of substance P in samples of normal and CF lung.

Sample	Normal	Sample	Cystic fibrosis.
301	<0.05	316	<0.05
365	<0.05	322	0.06
353	0.50	283	0.23
332	<0.05	327	<0.05
329	0.08	321	<0.05
302	0.06	325	0.20
		358	0.23
mean score± SEM	0.21±0.175		0.18±0.05

Statistical comparison of the results revealed no significant difference in the levels of VIP or substance P between tissue from normal patients and patients with CF

3.4.2. Spectrophotometric determination of the activity of serum butyrylcholinesterase

The activity of butyrylcholinesterase was measured in the serum from 21 patients in five groups:-

a. Asthma - symptomatic (4 patients)

b. Asthma - asymptomatic (4 patients)

c. Hyperreactive - +ve methacholine challenge (3 patients)

d. Hay fever - atopic (5 patients)

e. Controls - normal subjects (5 patients)

The rates of activity were measured over the first five minutes of the reaction i.e. the linear phase (for details of the calculation of rate of reaction see Methods, section 2.4.5). The rates are expressed as the number of moles of substrate hydrolysed per min per ml of serum $x10^{-3}$.

TABLE 45.

Butrylcholinesterase activity in samples of human serum.

Sample No.	Asthma (Symp)	Asthma (Asymp)	Hyper-Reactive	Hay Fever	Controls
1	1.192	1.025	1.145	1.032	1.144
2	1.029	1.167	1.146	0.898	0.901
3	1.444	1.129	0.938	1.396	1.180
4	1.115	0.837		1.223	1.288
5				0.805	1.094
	n=4	n=4	n=3	n=5	n=5

Comparison of the rates of reaction between the normal control group and the other groups revealed no significant differences.

3.5. SUMMARY OF RESULTS.

The types of autonomic nerves in the airways have been determined by studying the distribution of the neural marker PGP 9.5. to delineate the gross nerve supply, enzyme histochemistry for the cholinergic supply, glyoxylic acid-induced fluorescence and immunohistochemistry of tyrosine hydroxylase for the adrenergic supply and immunohistochemistry for peptides in the 'NANC' system.

The number of nerves stained with antibodies to PGP 9.5 in the airway epithelium and the distribution in tissue sections of substance P and CGRP were quantitatively assessed in normal, disease control ('young' and bronchiectatic tissue) and asthma and cystic fibrosis. The distribution of cholinergic, adrenergic, VIP and NPY nerves in the airways was assessed semi-quantitatively in normal, disease control and cystic fibrosis tissue, and the results expressed in an analysis of variance.

The results are also given for the determinations by radioimmunoassay of the levels of VIP and substance P in tissue samples from normal patients and patients with cystic fibrosis and of the biochemical determinations of the activity of butyrylcholinesterase in serum from normal, 'atopic' and asthmatic patients.

1. Normal lung; qualitative assessment.

i. There are nerves and neuroendocrine cells in the epithelium as shown by staining for PGP 9.5. The mean (±SEM) number of nerves per mm of airway epithelium in normal lung was found to be 1.58±0.17. Both substance P and CGRP are present in epithelium, bronchial smooth muscle and in association

with blood vessels, but are sparse. CGRP is present in some neuroendocrine cells. Radioimmunoassay has shown insignificant levels of substance P in 3 of the 6 'normal' samples analysed.

ii. There is positive stain for catecholamine in airway smooth muscle. The existence of a sympathetic component to airway smooth muscle is supported by the presence of strong staining for tyrosine hydroxylase in the smooth muscle and the presence of NPY-immunoreactive fibres.

There is dense staining for cholinesterase and VIP immunoreactivity in the bronchial smooth muscle.

iii. There are large amounts of butyrylcholinesterase in human lung in both the vascular and bronchial smooth muscle, the density increasing with increasing airway generation.

iv. In order of density of stain, the airway ganglia stain positively for cholinesterase, VIP, tyrosine hydroxylase, catecholamines, substanceP, CGRP and NPY.

v. There is a cholinergic supply to the bronchial and pulmonary vessels, albeit sparse. The most dense stains in association with airway vasculature were attributable to catecholamines, NPY, tyrosine hydroxylase and VIP.

2. Differences between airway levels: quantitative assessment.

Nerves were present to the level of the sub-segmental airways. None were detected in the alveoli. There was a significant decrease in the density of cholinergic innervation of bronchial smooth muscle, blood vessels and lamina

propria. However, the submucosal gland was consistently well innervated.
There were no significant differences in the aminergic innervation with increasing airway generation, except for a reduction in the incidence of stain in the cholinergic ganglia. However, the variablility of the score between different patients precludes any conclusion. In general, there was no reaction for catecholamines beyond the level of the sub-segmental airways. Where catecholamines were present, the density was consistent. There was significant variation in the density of stain between patients.
There was no trend toward decreased density of VIPergic innervation with increasing airway generation. However, the analysis of variance indicated significant differences between airway levels. This is probably a reflection of the variability of the samples.

3. Differences in disease: quantitative and semi-quantitative assessments.

i. Disease control groups

a. Bronchiectasis. The point counts indicated that bronchiectatic tissue had an increased area of submucosal gland in comparison to the normal, young and asthmatic tissue, but not in comparison to the cystic fibrosis tissue. The difference was not statistically significant.

The tissue showed a significantly lower score for all types of innervation in all the areas looked at in comparison to the normal tissue: exceptions; NPY in ganglia and pulmonary vessels

b. Young tissue. The point counts indicated that, in general, this tissue was not morphologically different to the 'normal' control group. In innervation, there were no consistent differences in comparison with the normal controls except in a reduction in the density of tyrosine hydroxylase in the

submucosal gland ($p<0.05$) and NPY in the vessels (bronchial, $p<0.05$ pulmonary, $p<0.01$). VIP scored significantly lower in gland ($p<0.01$) and in ganglia ($p<0.05$). The distribution of substance P and CGRP was no different.

ii. Disease groups.

a. Cystic fibrosis. The CF tissue had the highest proportion of submucosal gland of all the groups examined. However, the difference was not satistically significant and there were no other marked differences in the proportion of the tissue section occupied by any of the other effector structures.

There were no significant differences between the 'normal' group and CF in the density of stain of tyrosine hydroxylase, cholinesterase, substance P or CGRP in any of the effector structures observed. There was no difference in the NPY innervation of any area except the bronchial smooth muscle which showed a reduction in the level of NPY ($p<0.01$). Comparison of the CF tissue with the 'young' group revealed no difference in the NPY score in the smooth muscle.

A marked reduction was observed in the score for VIP in the CF tissue compared to the normal samples for submucosal gland and bronchial smooth muscle in benzoquinone fixed, frozen tissue and a reduction in VIP in the submucosal gland, bronchial smooth muscle and ganglia in formalin-fixed, paraffin-embedded tissue. Comparison of the CF tissue with the 'young' tissue revealed a significantly lower score for VIP in the submucosal gland but not in the smooth muscle. Comparison of the levels of VIP and substance P measured by radioimmunoassay revealed no differences in the levels of either peptide in comparison with normal tissue; 3 of the 7 cases of CF showed insignificant levels of substance P. The density of VIPergic

innervation was inversely correlated to the percent area of submucosal gland on the tissue section, and this correlation was significant at the 5% level (i.e. $p<0.05$). The number of nerves in the epithelium was significantly lower in CF compared to the normal tissue.

b. Asthma. The point counts indicated that the asthmatic tissue had a slightly larger proportion of bronchial smooth muscle than the normal or other groups of tissue, however this difference was not statistically significant. There were no other marked differences, in particular the area of the tissue section occupied by ganglia was no different in comparison with the normal tissue.

In the one case of fresh asthmatic tissue obtained, there were no apparent obvious differences in the distribution of markers for cholinergic, adrenergic, VIP or substance P nerves. The paraffin embedded tissue did not stain for CGRP or NPY, so no information is available on the distribution of these markers in asthma. Quantitative assessment of the distribution of substance P revealed no differences in the distribution in comparison with the normal control tissue, and the number of nerve fibres per mm length of epithelium was no different in comparison with the normals. One case out of 5 showed a hyperplasia of substance P nerves, so did one normal case. 2 of the 5 cases showed a hyperplasia and thickening of PGP 9.5 reactivity in the lamina propria. One of these cases (19/84) was also stained for substance P and showed a similar hyperplasia. There was no difference in the activity of butyrylcholinesterase in serum from asthmatic patients in comparison to normal patients and patients who displayed hyperreactivity.

CHAPTER 4.

DISCUSSION

4.1. THE DISTRIBUTION OF NEURAL MARKERS IN NORMAL LUNG.

In this section the results of the assessment of the distribution of neural markers in normal lung are discussed and the functional implications of the findings are considered, with specific reference to the questions highlighted in Chapter 1. i.e.:-

1.i. What are the nerve types and numbers in the airway lining epithelium?

ii. What are the roles of the 'sensory' neuropeptides (substance P & CGRP); do they have a motor influence in the human lung?

2. What is the extent of peptidergic innervation in airway submucosal gland?

3. Is there a sympathetic supply to bronchial smooth muscle?

4. What is the role of butyrylcholinesterase in the lung ?

5. What is the innervation of airway vasculature?

6. Are there neurotransmitters other than acetylcholine present in airway parasympathetic ganglia?

7. Are there differences in innervation at different airway levels?.

4.1.1. Epithelial innervation.

i. Number of nerves in surface epithelium.

In the present study, intra-epithelial nerves, stained for PGP 9.5, were counted in paraffin sections of formalin-fixed, human segmental airway. PGP 9.5 is exclusively localised to neuronal cytoplasm [Doran et al 1983] and has been shown to be a specific marker for vertebrate neurons and neuroendocrine cells [Thompson et al 1983]. Previously, there have been no studies that have attempted to quantify human airway epithelial nerves at

light microscopic level. The results of the present study have shown that the number of nerve fibres per mm length of normal human airway epithelium is less than that reported in ultrastructural studies [Laitenen 1985b] (approximately 2 nerves per mm compared to approximately 4 per mm respectively, for the equivalent airway level). This may be due to a difference in the method of counting the nerve fibres. At ultrastructural level, every individual nerve profile observed was counted, but this proved difficult at light microscope level because where many nerve fibres were grouped together, it was impossible to count them individually. Therefore, a method was adopted (for the purposes of comparison between disease states) whereby only those fibres running perpendicular to the basement membrane were counted, and this may have produced counts less than those produced by counting at ultrastructural level. Alternatively, Laitenen may have made an overestimate of the numbers of nerves, based on the small sample sizes necessited by quantification at ultrastructural level: Laitenen was only able to sample a total epithelial length of 5.63mm, whereas in the present study, the total length of epithelium measured in normal lung, was 220.2mm.

The number of nerves in human airway epithelium, demonstrated by either the present study or ultrastructural studies, is low in comparison to the number present in rat ($23mm^{-1}$ in main bronchus) [Jeffery and Reid 1973], or cat ($16.5mm^{-1}$ in the hilar region) airway epithelium [Das et al 1979]. By contrast, mouse tracheal epithelium lacks an intra-epithelial nerve supply [Pack et al 1980]. It is evident that wide species differences exist in the extent of epithelial innervation. The significance of this inter-species variation is not clear, but it should be a consideration in functional studies of airway reactivity when attempting to extrapolate the results of animal studies to man.

ii. The role of intra-epithelial nerves.

The distribution of nerve fibres in the epithelium demonstrated in the present study is in agreement with ultrastructural studies of intra-epithelial nerves which show axons penetrating the basement membrane and branching out within the epithelium. The nerve profiles seen at ultrastructural level contain neurotubules, mitochondria and occasionally vesicles, and tend to be located near the basement membrane, except in larger airways where some profiles are located close to the lumen [Laitenen 1985b, Jeffery 1987]. Since the ultrastructural observation of Rhodin in 1966 of a single nerve fibre (lacking neurosecretory vescicles) in human tracheal epithelium, there has been much debate about the sensory or motor role of intra-epithelial nerves. The rabbit and the goose have been used for physiological investigations into the control of epithelial goblet cell function as their large airways have goblet cells but few submucosal glands [Boyd 1972, Phipps et al 1977]. Evidence of a lack of a 'classical' motor innervation to rabbit airway epithelium has been provided by Peatfield, who was unable to demonstrate any effect on the release of radiolabelled glycoproteins from the airway epithelium by the administration of adrenergic and cholinergic agonists to isolated tracheal segments [Peatfield 1980]. This, however, does not discount the possiblity of a NANC mechanism. In the goose, there is some evidence for a 'classical' motor innervation in the airway epithelium [Phipps et al 1977]. In addition, functional studies in this species have shown that maximal concentrations of cholinergic and adrenergic antagonists fail to block the secretory response to electrical stimulation of parasympathetic nerves, indicating the presence of a NANC control mechanism [Phipps et al 1977]. However, in the healthy adult human, it has been shown that the volume of goblet cells approximates to $1/40^{th}$ of the total volume of the submucosal glands [Reid 1960]. Therefore, in contrast to the goose and rabbit, it is the

submucosal glands that are potentially responsible for the greater contribution to the secretion of respiratory tract mucus in man. In the present study, there was no evidence of cholinergic, adrenergic, VIPergic or NPY nerves in human epithelium, suggesting a lack of both 'classical' and NANC motor innervation. These findings are in agreement with those of other workers who have also found no structural evidence of a 'motor' innervation to epithelial cells [Fillenz and Woods 1970, Sheppard et al 1983, Laitenen 1985a].

In the present study, however, the presence of varicosities on intra-epithelial nerves stained with antibodies to PGP 9.5 suggested that these nerves might contain other neurotransmitters which could affect the function of the epithelium. Staining of alternate sections for PGP 9.5, substance P and CGRP has shown a very similar distribution in the epithelium and it is highly likely that a proportion of the nerves staining for PGP 9.5 contain substance P or CGRP, or possibly both. Co-localistion of substance P and CGRP has been reported in primary sensory neurons in guinea-pig bronchioles [Lundberg et al 1985a] and their distribution seen in the present study suggests that they are also co-localised in human airway epithelial nerves. However, it is not possible at light microscope level to determine whether two or more neurotransmitters are localised to the same nerve.

Physiological studies have demonstrated the presence of sensory nerves in mammalian airways and have indicated the close association of their endings with the airway epithelium [see Coleridge and Coleridge 1984, Widdicome 1981, Widdicome 1986]. The afferent nature of nerve fibres in rat airway epithelium has been demonstrated using cobalt chloride diffusion and precipitation techniques; the dye diffuses without synaptic interruption from the cut ends of pulmonary vagi to terminations in the bronchial epithelium [Hoyes et al 1982]. Surgical denervation studies suggest that cervical

vagotomy will cause degeneration of afferent fibres (whose cell bodies lie in the nodose ganglion) leaving intact the post-ganglionic parasympathetic motor fibres (whose cell bodies are in the airway wall): in the cat, 70-90% of airway intraepithelial nerves degenerate 5 days after vagotomy [Das et al 1979]. Capsaicin, an extract of hot peppers, is known to selectively activate a population of vagal C-fibre afferents in the airways when given acutely [see Coleridge and Coleridge 1984, Widdicome 1981], and cause local release of bioactive peptides. For example, after exposure to capsaicin, tachykinin- [Lundberg et al 1984a] and CGRP-immunoreactive nerves [Cadieux et al 1986] disappear from the airways. Histochemical and biochemical evidence, however, suggests that cholinergic, VIPergic and aminergic nerves remain after capsaicin treatment [Jansco et al 1968, Lundblad et al 1983]. The involvement of substance P in sensory nerves has been demonstrated in the guinea pig by the activation of vagal C-afferents by local application of capsaicin to the vagus nerve. This leads to a reduction in substance P-immunoreactivity in the airways. The remaining substance P reactivity disappears after removal of the stellate ganglion suggesting that substance P-immunoreactive sensory nerves also enter the lung via sympathetic pathways and have a spinal sensory origin in the guinea-pig. [Lundberg et al 1983a, Saria et al 1985]. The present study has demonstrated substance P-immunoreactivity in human airway cholinergic ganglia suggesting that one route by which substance P nerves may enter the human lung is via parasympathetic pathways.

It has been suggested that stimulation of sensory nerve endings by irritant chemicals could lead to antidromic release of mediators from sensory nerves [Lundberg and Saria 1983] and that tachykinins (including substance P) and CGRP may be involved in local axon reflexes [Barnes 1986]. This concept is discussed further in sections 1.3 and 1.8. Little is known about the actual

effects of the local release of substance P or CGRP from nerves within the human airway epithelium. Substance P has been shown to produce a transient rise in epithelial ion transport in the canine trachea in vitro [Al-Bazzaz and Kelsey 1982] and therefore has a potential role in the control of epithelial permeability.

In the present study, staining with PGP 9.5 has revealed the presence of single neuroendocrine cells in the epithelium. However, no groups of neuroendocrine cells resembling neuroepithelial bodies were found in any of the samples examined. The existence of neuroepithelial bodies in adult human airway epithelium has been reported [Lauweryns and Godeeris 1975, Cutz et al 1981, Cutz et al 1984], however they are infrequent compared to human foetal lung [Lauweryns and Peuskens 1972, Cutz et al 1984] and to other species e.g. rabbit [Lauweryns and van Lommel 1987].

The present study has demonstrated the presence of CGRP-immunoreactivity in epithelial neuroendocrine cells, in agreement with findings of some workers [Springall et al 1984], but contrasting with others [Palmer et al 1987]. The sensory origin of CGRP has been demonstrated in the rat by experiments in which neonatal pretreatment with capsaicin leads to a loss of CGRP from both nerves and neuroendocrine cells in the lung [Cadieux et al 1986]. The changes in number of neuroendocrine cells following vagal ligation suggest that these cells may be under neuronal influence [Lauweryns et al 1985, Cadiuex et al 1986]. Nerves have been reported in association with neuroendocrine or amine-containing cells in human lung before [Lauweryns and Peuskens 1972] and the present study has shown a close association between nerves and occasional, single neuroendocrine cells (stained for PGP 9.5) in the airway epithelium [see plate 5, fig.15].

The observations of other workers suggest a sensory role in the detection of hypoxia for these cells. For example, in the rabbit, they secrete dense-

cored vesicles in response to hypoxia [Lauweryns and Cokelaere 1973, Lauweryns et al 1983] and this response is modified by infranodose vagotomy [Lauweryns and van Lommel 1986]. There is also hyperplasia of neuroendocrine cells in response to to chronic hypoxia [Taylor 1982].

In the present study, the number of neuroendocrine cells staining for CGRP was not as great as that staining for PGP 9.5. suggesting that there may be two or more populations of cells with distinct contents. Bombesin, calcitonin and leu-enkephalin have all been demonstrated in neuroendocrine cells in human lung [Cutz et al 1981]. The low proportion of neuroendocrine cells that stained for CGRP in the present study, could also represent the loss of CGRP in response to the hypoxia of the lung during resection of the tissue.

4.1.2. Is there a sparcity of substance P and CGRP in effector structures in human airways?.

In the present study, although substance P and CGRP were frequently found in the airway epithelium and airway ganglia, there appeared to be a sparcity of them in bronchial smooth muscle, submucosal gland and around airway vasculature. In addition to the immunohistochemical findings, radioimmunoassay of substance P in normal human airway revealed that in 3 out of 6 patients, the levels were below the detection limit of the assay. However, detectable amounts were found in the airways of the remaining patients. The levels found in the present study are lower than those reported by other workers (an average of 0.2 picomoles per gram wet weight of tissue compared with 3.0 pmoles per gram reported by Lundberg et al [1984]). However, the levels of substance P in human bronchi, found in both the present study and that of Lundberg and colleagues [1984], are low in comparison to the levels in guinea-pig lung [4.1 - 12.0 pmoles per gram for

the equivalent region of lung, Ghatei et al 1982]. These observations are supported by the finding of the present study of an abundance of substance P-immunoreactive nerves in control guinea-pig tissue compared to a lack in human airways. Similarly, low levels of CGRP have been reported in human lung (approximately 1.5 pmoles per gram in bronchus, Palmer et al 1987) compared to rat (33-37 pmoles per gram in bronchus, Cadieux et al 1986).

It has been suggested that the observed lack of both substance P and CGRP in 'normal' human lung is due to the tissue having been obtained from elderly patients with a history of smoking, undergoing resection for carcinoma [Barnes 1987]. It is possible that some reduction occurs with age or smoking. However, in the present study, the young control group (none of whom had a history of smoking) showed a similar sparcity of substance P and CGRP-immunoreactivity. The observation in the present study, that substance P and CGRP are frequently found in the epithelium and airway ganglia but not in association with bronchial smooth muscle or airway vasculature, indicates that the sparcity is confined to specific tissues and is therefore more likely to be real. The peptides may have been lost during tissue processing, although the observations of a lack of substance P and CGRP in the rapidly benzoquinone-fixed tissue, which is not subjected to the same rigours of processing as the paraffin-embedded tissue, suggests that this is not the case; control tissue from the guinea-pigs used herein was always positive for both substance P and CGRP whether processed to wax or fixed in benzoquinone and then frozen. An alternative explanation is that the peptides were lost due to post-resection autolysis. However, in the present study, tissue continued to exhibit a positive reaction for both substance P and CGRP for up to 72 hours before fixation. Therefore, the sparcity of substance P and CGRP observed in the present study is probably due to a

real sparcity and cannot be fully explained by a reduction due to autolysis, or other factors such as the age of the patient.

4.1.3. Do substance P and CGRP have a 'motor' function in human airways?

The findings herein and those of other workers of low levels of substance P and CGRP in the human lung, in areas other than the epithelium and airway ganglia, raises questions about their putative 'motor' function. Substance P and CGRP may affect target tissues by retrograde antidromic release from sensory fibres directly innervating the effector structure, or by neuromodulation of parasympathetic transmission through airway ganglia [Barnes 1986c].

Neuromodulation of parasympathetic transmission is likely in view of the finding herein and of other workers of substance P and CGRP-immunoreactivity in human airway ganglia [Lundberg et al 1984, Palmer et al 1986]. The findings of a sparcity of substance P and CGRP in effector structures, however, contrasts with functional evidence. For example, substance P has been shown to stimulate mucus secretion in isolated human airways [Barnes et al 1986]. Both substance P and CGRP exert vasodilatory effects in man; substance P is a potent peripheral vasodilator [Fuller et al 1987] and CGRP has been shown to relax coronary artery in vitro [Franco-Cereceda and Lundberg 1987]. However, there is little information on the effect of substance P or CGRP on human pulmonary vasculature. Studies on cat trachea have shown that vagal nerve stimulation in the presence of a ganglionic blocking agent (hexamethonium) still induces vasodilation in cat tracheal mucosa [Lundblad et al 1983, Martling et al 1987] suggesting antidromic stimulation of sensory nerves is occuring. Substance P is also a

very potent stimulator of protein extravasation in animal airways via increased permeability of post-capillary venules [Lundberg and Saria 1982, Lundberg et al 1983c, Persson 1985]. CGRP, however, does not have the same effect [Lundberg et al 1985], but may potentiate the effects of substance P via its vasodilator activity [Brain et al 1985].

Infused substance P has profound cardiovascular effects, but little effect on airway smooth muscle in human subjects [Fuller et al 1987]. However, in vitro studies have shown that substance P constricts human airway smooth muscle [Fuller et al 1985] but other tachykinins, especially neurokinin A and eledoisin, are much more potent [Karlsson and Persson 1985, Martling et al 1987, Palmer et al 1986]. Inhalation of capsaicin in human subjects induces coughing, but only a transient bronchoconstriction [Fuller et al 1985]. In vitro, however, capsaicin does produce bronchoconstriction which is inhibited by substance P antagonists [Lundberg et al 1983]. However, the specificity of substance P anatagonists has been questioned and it is possible that they may exert a non-specific neurodepressant action [see Karlsson et al 1984]. Tachykinins are considered to exert their effects on target organs via different types of receptors [see Buck and Burcher 1986]; substance P receptors have been demonstrated in human lung on epithelium, submucosal glands, and on bronchial and vascular smooth muscle [Carstairs and Barnes 1987]. In the guinea-pig trachea, studies on phosphinositide turnover in response to tachykinins have shown that the greatest stimulation in the epithelium was elicited by substance P, and by neurokinin A in the bronchial smooth muscle [Grandordy et al 1987], suggesting that substance P is more active in epithelium than in smooth muscle.

By contrast, CGRP has been shown to exert potent bronchoconstrictor effects on in vitro preparations of human bronchial smooth muscle [Palmer et al 1987]. CGRP receptors have also been localised in human lung [Carstairs

1987].

Therefore, there is some functional evidence to suggest substance P and CGRP have a 'motor' function in human airways. The presence of both substance P and CGRP receptors in the human lung would seem to support an in vivo role for these peptides [Carstairs and Barnes 1986a, Carstairs 1987]. However, the findings of a lack of both peptides in bronchial smooth muscle and around airway blood vessels in the present study, would seem to indicate more of an indirect role via neuromodulation of transmission through airway ganglia. This is discussed further in section 1.8.

4.1.4. The control of tracheobronchial submucosal glands.

The presence of acetylcholinesterase in fibres located around submucosal glands, demonstrated in this study, is consistent with the functional evidence of cholinergic stimulation of gland secretion [Nadel et al 1985]. The present study has also shown a consistent aminergic innervation of submucosal gland, although the density of innervation was less than the cholinergic supply observed using acetylcholinesterase histochemistry (a mean score of aproximately 2 compared with 4 for the cholinergic supply). Functional studies indicate that both alpha and beta adreno-receptor agonists increase the output of secretory glycoproteins from human bronchi in vitro [Phipps et al 1980, Peatfield and Richardson 1982]. Evidence for NANC influence on gland secretion has been presented in the cat and the ferret [Phipps et al 1980, Peatfield and Richardson 1983, Gashi et al 1986, Peatfield et al 1983]. In the human lung, evidence has been presented that suggests NANC mechanisms are not involved the control of submucosal gland function [Baker et al 1984]. However, the results of these experiments were too variable to allow definite conclusions.

In the present study, VIP was shown to supply both serous and mucous components (as determined by staining alternate sections with AB-PAS, appendix 3) of the submucosal gland acini with a dense network of fibres. VIP has been shown to cause a dose-dependent inhibition of both baseline and methacholine stimulated output of glycoconjugates and lysozyme from human submucosal gland in vitro. This was shown to be connected with an inhibitory effect of VIP on macromolecular discharge by both serous and mucous cells [Coles et al 1981]. There is evidence in the ferret trachea that VIP has a differential effect on serous and mucous cells, inhibiting the secretion from mucous cells produced by methacholine and enhancing the secretion from serous cells produced by phenylephrine [Webber and Widdicome 1987]. However, no differential innervation of serous and mucus cells was found in the present study. In support of this observation, there appears to be no difference in the distribution of VIP receptors to serous or mucous acini [R. Sharma, personal communication]. Other workers have shown that VIP stimulates Cl^- and water transport across intestinal [Kreijs et al 1980] and tracheal epithelium [Nathanson et al 1983] It is therefore possible that VIP may act in the human submucosal gland by modulating the proportions of the constituents of mucus.

The present study has shown an absence of NPY in the submucosal gland; this finding contrasts with those of other workers [Sundler et al 1986]. There is at present, however, no evidence of the effect of NPY on gland secretion.

4.1.5. The control of bronchial smooth muscle.

In agreement with earlier studies [Laitenen 1985a, Sheppard et al 1983], application of acetylcholinesterase histochemistry in the present study has revealed a dense supply of cholinergic nerves to human bronchial smooth

muscle. Some of the stain, attributable to acetylcholinesterase by the use of specific inhibitors, was diffuse rather than localised to nerve fibres. This weak diffuse stain may be a diffusion artefact, although it should be noted that not all acetylcholinesterase activity is localised to cholinergic nerves. Studies on skeletal muscle have shown intracellular cholinesterase activity in association with the myosin filaments and it has been suggested that, in this location, the cholinesterase may play a role in regulating the contraction cycle of the muscle as very high concentrations of acetylcholine inhibit ATPase [Varga and Szoor 1971].

The normal resting tone of the airways is maintained by vagal efferent activity [Nadel 1980]: electrical stimulation of cholinergic nerves is inhibited by muscarinic receptor antagonists and potentiated by cholinesterase inhibitors [Nadel 1980, Nadel and Barnes 1984].

The present study has also shown a high density of VIP-immunoreactivity in human bronchial smooth muscle, which was present to the level of the bronchioles (airway levels III-IV). VIP has been demonstrated by other workers in human bronchial smooth muscle [Laitenen 1985a, Sheppard et al 1983] and the role of VIP in the airways has recieved much attention. VIP causes human bronchial smooth muscle to relax in vitro and is 50 times more potent in this than isoprenaline [Palmer et al 1986a, 1986c]. However, in vivo, inhaled VIP does not cause the same degree of bronchodilation as that observed in vitro, in subjects who responded to a β-agonist. The lack of effect may be due to the breakdown of VIP before it reaches the smooth muscle [Barnes and Dixon 1984]. Similarly, given by infusion, VIP has a bronchodilatory effect, but its effects on blood vessels prevent accurate study of its effects on the airways [Morice et al 1984]. Howewever, the presence of significant amounts of VIP, coupled with the presence of VIP

receptors [Carstairs and Barnes 1986], suggests that VIP may have a role human bronchial smooth muscle in vivo.

Is there a sympathetic supply to human bronchial smooth muscle? The presence of catecholaminergic nerves in human bronchial smooth muscle has long been a matter for debate: some workers claim an absence of aminergic nerves [Richardson 1979, Richardson and Beland 1976] and others claim that they are present [Sheppard et al 1983, Laitenen 1985a, Laitenen et al 1985, Pack and Richardson 1984, Partanen et al 1982]. The general concensus of opinion is that human bronchial smooth muscle receives a very sparse aminergic innervation. The present study has shown that aminergic nerves are present in human bronchial smooth muscle, but in agreement with the majority of authors, they were infrequent and at best, very sparse in comparison to the cholinergic supply (a mean score in the present study of 0.6 compared with 4.7 for the cholinergic supply). In support of these observations, ultrastructural studies have demonstrated a sparcity of adrenergic nerve varicosities adjacent to smooth muscle cell bundles [Daniel et al 1986]. One of the problems found in the present study associated with the delineation of aminergic innervation, is the susceptibility of noradrenaline to rapid depletion on exposure of the tissue samples to air. This study has shown that tissue left exposed to the atmosphere for more than 4 hours loses most of its catecholamine content and this disappears altogether from samples stored at $-70^{o}C$ for longer than 2-3 months. Despite incubation of the samples with a metabolic precursor (L-DOPA), in an attempt to replenish the catecholamine content of the tissue, the innervation observed in bronchial smooth muscle remained sparse. This susceptibility to post-resection depletion, coupled with the observations of other workers that sympathetic activity decreases with the age of the patient [Christensen 1982] could partly

explain the apparent sparcity of adrenergic innervation of human bronchial smooth muscle found in both the present study and those of other workers.

The demonstration herein of positive catecholamine fluorescence coupled with large amounts of positive diffuse stain for tyrosine hydroxylase in the bronchial smooth muscle contrasts with previous suggestions of a lack of a functional sympathetic influence [Doidge and Satchell 1982, Davis et al 1982a, Taylor et al 1984]. It should be noted, however, that many studies on small airways function have been carried out on lung parenchymal strips. Such results do not necessarily represent changes in peripheral airways smooth muscle, but rather vascular smooth muscle, which is more abundant in peripheral lung [Finney et al 1985]. More recently, evidence of a functional sympathetic innervation has been supplied by Davis and Kannan (1987) using in vitro preparations of human peripheral bronchi. They found that noradrenaline is released by electrical field stimulation and tyramine. Also, the relaxation response, induced by exogenously applied noradrenaline, was inhibited by neuronal uptake blockade with cocaine [Davis and Kannan 1987]. However, other studies have shown that neuronal uptake blockade by cocaine has no effect on the noradrenaline dose response curve [Zaagsma et al 1984].

It has been postulated that β_1-adrenoreceptors are targets for neurally released noradrenaline, and β_2-adreoreceptors are activated by the 'neurohormone', adrenaline. This hypothesis implies a relationship between the number of β_1-receptors and the density of aminergic innervation. Evidence for this has been provided in studies on cat tracheal smooth muscle, which has a dense aminergic innervation [Silva and Ross 1974], and the relaxation response of which is mediated predominantly by β_1-receptors [O'Donnell and Wanstall 1983]. Zaagsma and colleagues have shown that the relaxation response of bronchial smooth muscle in guinea-pig and human airways is

mediated by β_2-receptors [Zaagsma 1983]. β_1-receptors are present in human lung, however, they are located mainly on the alveolar walls where their function is unknown [Carstairs et al 1985]. In view of the sparse adrenergic innervation of human smooth muscle, it has been suggested that circulating catecholamines may be important in regulating bronchial smooth muscle tone via β_2-receptors present on the bronchial smooth muscle [Carstairs et al 1985]. Plasma noradrenaline is derived mainly from an overspill of sympathetic nerve activity [Brown et al 1981]. However, infusions of noradrenaline at normal physiological concentrations have no effect on airway function in normal individuals [Berkin et al 1985]. This suggests that noradrenaline does not in fact act as a circulating hormone.

In the absence of convincing evidence of a functional noradrenergic innervation, it is possible that the aminergic nerves in the smooth muscle are involved in neuromodulation for other neurotransmitters. This hypothesis is discussed further in section 1.8.

The presence of NPY in human bronchial smooth muscle was demonstrated in the present study. There is evidence from other species which indicates that NPY is located in sympathetic nerves [Sheppard et al 1984b, Sundler et al 1986] and interacts with adrenergic mechanisms [Lundberg et al 1982, Lundberg and Stjarne 1984]. In the present study, NPY was observed in varicose fibres throughout the airway smooth muscle, occurring more frequently than aminergic fibres. There is functional evidence for the existence of both pre- and post-junctional receptors for NPY on rabbit vascular smooth muscle [Wahlstead et al 1986]. In the context of bronchial smooth muscle, this suggests that as well as exerting a modulatory influence on the release of noradrenaline, NPY may act as a neurotransmitter in its own right.

In the present study, the finding of a consistent presence of NPY fibres in

the bronchial smooth muscle suggests a possible alternative neurodilator mechanism to noradrenaline. No functional studies have been done, however, on the effects of NPY on human bronchial smooth muscle. In the guinea-pig, NPY has no direct effect on trachealis muscle in vitro. It does, however, produce a concentration dependent inhibition of the cholinergic component of the response elicited by electrical field stimulation, but has no effect on exogenously applied acetylcholine [Stretton and Barnes 1988]. This evidence indicates a prejunctional modulation by NPY of cholinergically induced smooth muscle contraction.

4.1.6. The role of butyrylcholinesterase.

The present study has shown intense, diffuse staining for butyrylcholinesterase (BuchE) in human airways, located mainly in bronchial smooth muscle and, to a lesser extent, in the smooth muscle of pulmonary vessels related to peripheral airways (levels IV and V). The role of BuchE is unknown, but several observations indicate it may be of importance. According to Augusstinson (1968) BuchE evolved separately from acetylcholinesterase (AchE) and does not represent a less efficient precursor of AchE. As the action of acetylcholine is to change membrane permeability to electrolytes, many studies on the non-neural role of both AchE and BuchE have centred around their possible function in controlling membrane permeablility. Both AchE and BuchE are associated with many membranes across which the transport of water and ions takes place e.g. salivary glands, sweat glands and glands of the aliamentary tract [see Silver 1974]. It has been suggested that the presence of large amounts of AchE on erythrocyte membranes is indicative of a role in the control of red cell permeablility [Grieg and Holland 1949a]. However, there is no acetylcholine and no choline

acetyltransferase in red blood cells [Hebb 1957], and hence no convincing evidence of a role for acetylcholinesterase in changing membrane permeability in these cells [Thompson and Whittaker 1952].

Studies on the role of BuchE have centred on its effect on the permeability of the blood/brain barrier and on vascular permeability, primarily because of its location at the relevant sites [Grieg and Holland 1949b]. A typical example is found in the work of Strickland and Thompson [1954, 1955] who studied the leakage of potassium from slices of chicken brain and found that eserine, 'DFP' and neostigmine (all potent inhibitors of cholinesterase) increased this leakage. However, the concentration of inhibitor required to produce this effect was far higher than that required to inhibit the esterase activity of BuchE. Both AchE and BuchE have been shown to hydrolyse substance P in vitro [Chubb et al 1980] and in the absence of any information about its physiological function, a role for BuchE as an endopeptidase in vivo has been suggested [Das et al 1982]. It has been shown that the peptidase site in the BuchE molecule is distinct from the esterase site and that high concentrations of eserine (500 times higher than is required to inhibit the esterase activity) do not affect the peptidase activity [Chatonnet and Masson 1984]. In view of the role of substance P in increasing vascular permeability and the ability of BuchE to hydrolyse substance P, it is tempting to speculate that BuchE has a role in this context.

4.1.7. The control of airway vasculature.

Blood vessels supplying the bronchial wall arise from the laryngeal and bronchial arteries [Berry 1935]. The arteries in the peribronchial tissue give rise to smaller branches which supply the submucosa and form venous

plexuses there [Daly and Hebb 1966]. The gross supply of nerves to the airway walls divides on entering the lung to give peribronchial and perivascular plexi [Spencer and Leof 1964, Fischer 1964, Nagaishi 1972]. The blood vessels, therefore, provide a pathway for the nerves to the airway wall. The present study has shown that no neural markers are found in association with pulmonary vessels in peripheral lung, and that the most dense innervation is around smaller, bronchial arterioles on the mucosal side of the cartilage. This is consistent with the observation that in most vascular beds the pre-capillary resistance vessels are the small arteries, arterioles and pre-capillary sphincters. These vessels are responsible for the largest fraction (>80%) of the total vascular resistance [Rosell 1978]. In the present study, the density of catecholamine fluorescence around bronchial vessels was significantly higher ($p<0.01$) than that observed around larger pulmonary vessels. Staining with tyrosine hydroxylase also indicated a greater density of aminergic nerves around bronchial vessels compared with pulmonary, but this difference was not statistically significant.

In the present study, aminergic nerves, delineated by both catecholamine fluorescence and tyrosine hydroxylase, were the most prevalent, compared with the other neural markers, around smaller bronchial vessels. In support of this observation, physiological studies indicate that a major controlling influence of the tracheobronchial vascular bed is via sympathetic alpha adrenoceptors which mediate vasoconstriction [Daly and Hebb 1966, Lung et al 1976].

The present study has shown that NPY is located in approximately equal proportions around both bronchial and pulmonary vessels. NPY is a potent vasoconstrictor of cerebral and coronary vessels [Edvinsson et al 1983, Gray and Morely 1986, Franco-Cereceda and Lundberg 1987]. A recent study has shown that local infusion of NPY into the human brachial artery causes

reduced forearm blood flow [Pernow 1988]. NPY also causes vasoconstriction in canine tracheal vasculature [Laitenen et al 1987a]. It therefore probably acts as a vasoconstrictor in the human lung.

Reports of the presence of cholinergic nerves around pulmonary and bronchial vasculature are contradictory [see Partanen et al 1982, Laitenen 1985a]. The present study has shown a very sparse acetylcholinesterase staining around small bronchial arterioles and no evidence of its staining around larger bronchial or pulmonary vessels, especially in peripheral airways (airway levels IV and V). Acetylcholinesterase in this location may not necessarily be indicative of a cholinergic nerve supply, as it has been shown to hydrolyse substance P [Chubb et al 1980]. Cholinergic influence over vasculature has been shown in dog lung where cholinergic stimulation results in a dilatory response which is partly reduced by atropine. The vasodilation is partly abolished by hexamethonium (which blocks transmission through parasympathetic ganglia) [Laitenen et al 1987b]. This suggests that the hexamethonium-sensitive response is attributable to preganglionic parasympathetic stimulation and the hexamethonium-resistant component suggests antidromic stimulation of sensory nerves may be taking place [Lundblad 1984]. However, this study has demonstrated an extremely infrequent incidence of immunoreactivity for the 'sensory' neuropeptides substance P and CGRP in association with human airway vasculature, a finding which contrasts with the observations that both have potent peripheral vasodilatory effects in man [Brain et al 1985, Eklund et al 1977, Fuller et al 1987]. Little is known, however, of the effects of substance P or CGRP on human pulmonary vasculature.

In contrast to the cholinergic supply, VIP has been shown herein to be present in relatively high density around both pulmonary and bronchial vessels. VIP is a potent vasodilator of canine tracheal vasculature, being

more powerful than the classic mediators histamine and methacholine [Laitenen et al 1987a]. It has also been shown to relax human pulmonary vessels in vitro and the response is independent of endothelial cells [Greenberg et al 1987]. This indicates that VIP acts directly on the vascular smooth muscle. VIP receptors have been demonstrated on human vascular smooth muscle [Carstairs and Barnes 1986] and it is therefore probable that VIP is an inhibitor of airway vascular smooth muscle tone in vivo.

4.1.8. Airway ganglia.

The finding of several different putative peptidergic neurotransmitters as well as evidence of a classical sympathetic component in the airway 'parasympathetic' ganglia in the present study, has widespread functional implications. The demonstration in the present study of an aminergic component and a positive immunoreaction for substance P in human airway ganglia has previously been reported [Richardson and Ferguson 1979, Lundberg et al 1984]. Other workers have reported an absence of NPY- immuno-reactivity in the airway ganglia [Sheppard et al 1984b], a finding that contrasts with that of the present study.

In the present study, acetylcholinesterase was found to be located almost exclusively in association with the nerve cell bodies of the ganglia. VIP was located in fibres around the nerve cell bodies, whereas catecholamines, NPY, substance P and CGRP were located in punctate foci between the nerve cell bodies. Butyrylcholinesterase was located in diffuse stain around the edge of the nerve cell bodies in a similar area to the location of the peptides. The loci of stain could represent terminations or cross-sections of pre- or post-ganglionic nerve fibres which innervate the effector structures. The findings suggest the possibility of pre-ganglionic aminergic and peptidergic modulation

of transmission through parasympathetic ganglia. So far, few physiological studies of ganglionic function have been done in human airways. In ferret trachea, post-ganglionic stimulation has a greater effect on airway smooth muscle activity than pre-ganglionic stimulation, indicating that some filtering takes place in the ganglia [Skoogh 1983]. Direct recording from ferret ganglionic neurons has demonstrated that noradrenaline inhibits neurotransmission via alpha adreno-receptors [Baker et al 1983].

A high density of VIP in the airway ganglia is reported in the present study. VIP has been shown to enhance cholinergic transmission in some ganglionic synapses [Kawatani et al 1985].

Electrophysiological studies on ferret airways have indicated that airway ganglia may receive an afferent input [Coburn 1984] and this is probably similar to the afferent input to intramural ganglia of the gut. Ganglionic neurons in the myenteric plexus have been demonstrated to generate signals independent of the central nervous system, indicating that independent, local reflexes can occur [Wood 1984]. However, there is no evidence at present that this occurs in peripheral airway ganglia. The findings herein of both substance P and CGRP in human airway ganglia imply an afferent input. The potential exists for a modulatory effect of substance P in cholinergic ganglia as it has been shown to block cholinergic nicotinic receptors [Belcher and Ryall 1977]. In the apparent absence of any significant direct innervation of human airway effector structures by substance P or CGRP, their presence in the airway ganglia may represent the major site of influence of these 'sensory' neuropeptides over human airway function.

There are further indications of integration between different nerve types: the findings herein of a close relationship between the distribution of acetylcholinesterase and VIP, catecholamine and NPY, and SP and CGRP,

suggests that the transmitters may be co-localised within the same nerve terminal. However, at light microscope level it is not possible to distinguish whether two or more neurotransmitters are localised to the same nerve. Ultrastructural studies have indicated that VIP and acetylcholine may be co-localised in nerves in human airways [Laitenen 1985a]. The ultrastructure of a nerve profile is considered to relate to the neurotransmitter it contains [e.g. Grillo 1966]. However, Gibbins has stated that there are no ultrastructural differences between 'cholinergic' and NANC autonomic nerves on the basis of the size of vesicle, or the proportions of the different types of vesicle [Gibbins 1982]. Therefore it may not be possible to distinguish the type of nerve, even at ultrastructural level, without the use of specific markers such as antibodies to the neurotransmitters.

Co-existence of several different neurotransmitters within the same nerve was suggested by Cook and Burnstock in 1976, and the concept of co-transmission, first suggested by Burnstock in 1976, is now widely accepted. Different neurotransmitters, released from the same nerve, or from nerves in close apposition to one another may produce pre-synaptic modulatory effects. The findings in the present study of aminergic nerves in human airway smooth muscle, coupled with evidence of a lack of a direct functional role for these nerves [Taylor et al 1984, Richardson and Beland 1976], suggests that they may instead be involved in modulation of cholinergic transmission. Cholinergic nerve varicosities have been shown to have receptors for several substances, including noradrenaline and adenosine, activation of which leads to modulation of acetylcholine release [Vizi 1979]. In canine bronchial smooth muscle, transmission at the pre-junctional terminal of parasympathetic nerves is reduced by sympathetic nerves stimulation and β-receptors are implicated [Vermiere and Vanhoutte 1979]. However, in man, Davis and Kannan [1987] found no evidence for sympathetic modulation of

cholinergic nerves in tracheal smooth muscle. There is, however, some circumstantial evidence for sympathetic modulation in more peripheral bronchi. For example Davis and Kannan report an attenuation of the contractile response to electrical field stimulation by a concentration of isoprenaline that causes insignificant attenuation of the response to exogenously applied acetylcholine. This indicates that some pre-synaptic sympathetic modulation of cholinergic function may occur.

VIP has been demonstrated in cholinergic neurons in the rat and in cat exocrine glands [Lundberg et al 1979, Lundberg 1981] and evidence has been presented of an interaction between acetylcholine and VIP. For example, it has been shown that VIP enhances the affinity of acetylcholine for muscarinic receptors [Lundberg et al 1980], and that the release of VIP and its binding affinity for VIP-receptors are increased by pre-treatment with atropine [Hedlung et al 1983]. Nerves releasing both VIP and acetylcholine have been demonstrated in salivary gland and it appears that different quantities of VIP and acetylcholine are released at different nerve discharge frequencies [Bloom and Edwards 1980, Lundberg et al 1979].

There is evidence to suggest that NPY is co-localised with noradrenaline in sympathetic neurons [Lundberg et al 1982, Sundler et al 1986]. NPY depresses the nerve-stimulated release of noradrenaline via a pre-junctional action [Lundberg et al 1982] and potentiates vasoconstriction in response to noradrenaline [Ekblad et al 1984]. There is also evidence that NPY may have pre-synaptic modulatory effects on cholinergic neurotransmission in guinea-pig trachea [Stretton and Barnes 1988].

Substance P and CGRP have been shown to co-exist within the cell bodies of sensory ganglia [Lundberg et al 1985a] and in afferent terminals in guinea-pig airways [Martling et al 1986]. In the guinea-pig trachea, substance P increases cholinergic transmission when post-ganglionic nerves are

stimulated with submaximal voltage [Barnes et al 1987] and in the rabbit, substance P has been shown to increase the release of acetylcholine from cholinergic nerves [Tanaka and Grundstein 1984]. However, the present study has shown no particular asssociation between acetylcholinesterase and substance P, except in the airway ganglia.

In summary, structural evidence indicates the potential for neuromodulatory effects between different neurotransmitters, either by modulation of transmission through airway ganglia or by pre-synaptic effects of co-released transmitters. However, demonstration of the existence of such effects in human airways awaits further physiological investigation. Hypothetical representations of efferent and afferent pathways to human airways, based on the findings of the present study, are presented in figures 4.1 and 4.2.

4.1.9. Are there differences in innervation at different airway levels?

The present study has demonstrated the presence of the neural marker, PGP 9.5, to the level of the subsegmental airways, beyond which there was no evidence of nerves. This finding contrasts with that of previous work which claims the presence of large numbers of nerves in peripheral lung [Spencer and Leof 1964]. However, the silver staining methods used by these authors are capricious, and it is now generally accepted that these methods stain reticular tissue and are therefore not reliable for the identification of nerves. In support of the present study, a study at ultrastructural level has shown a sparcity of nerves in human alveolar walls: nerves were found in only 3 of 16 patients examined [Fox et al 1980]. These nerves were unmyelinated and agranular, which suggests a sensory function [King et al 1974]. Information about afferent nerve terminals in human airways is limited. However, three different types of ending have been described;

Fig. 4.1. Hypothetical diagram of efferent pathways to the airway wall

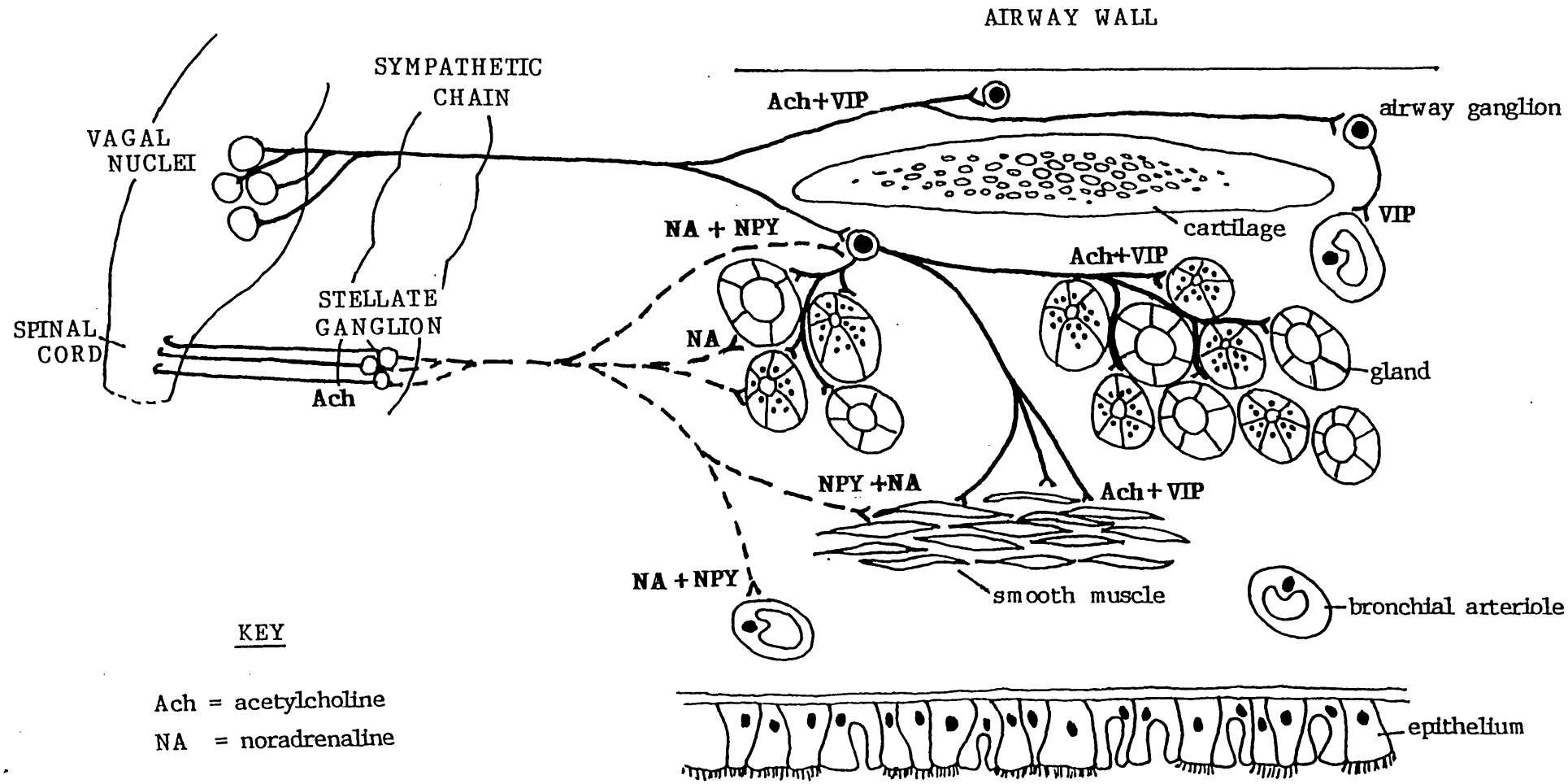
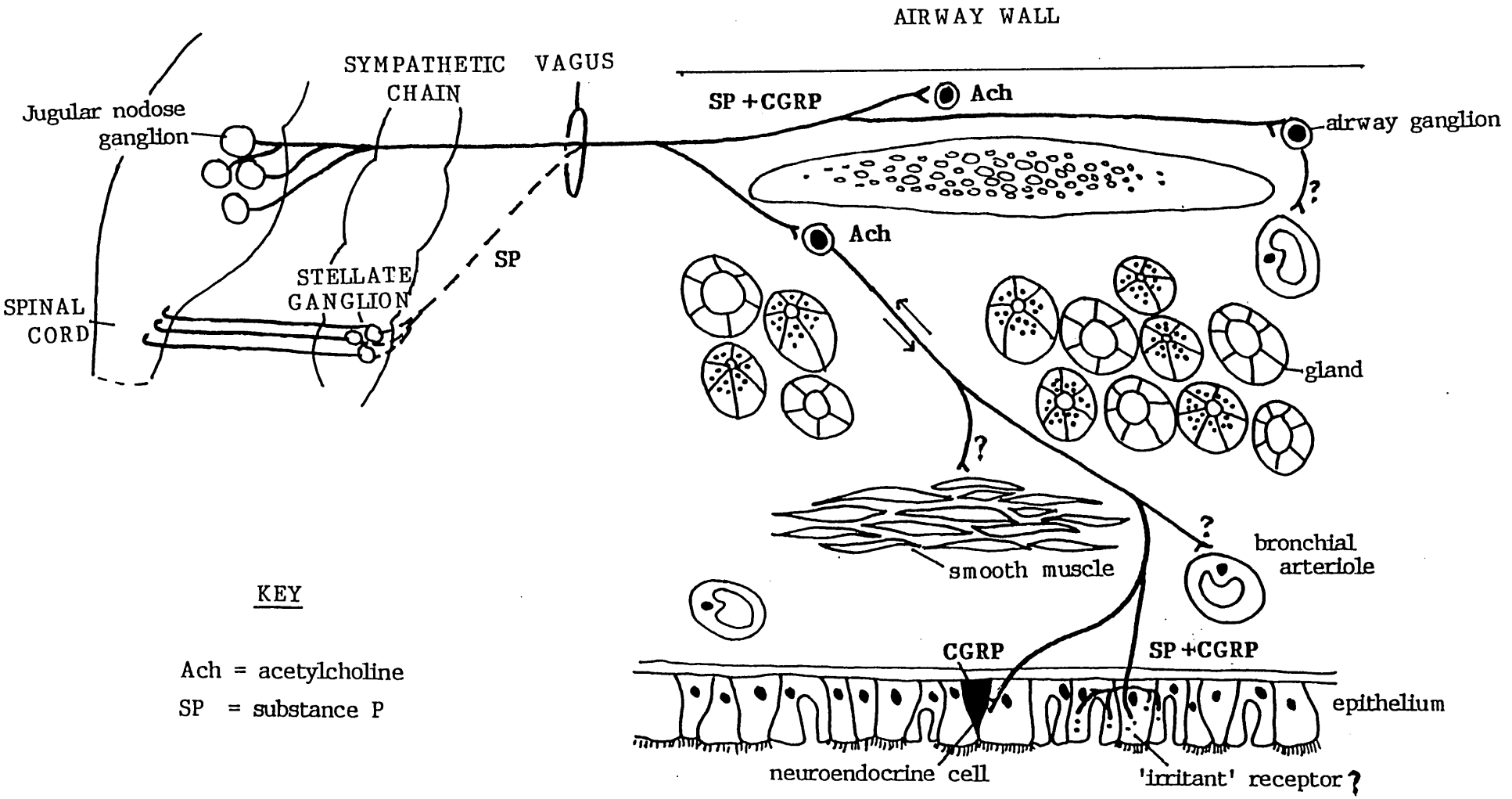


Fig. 4.2. Hypothetical diagram of afferent pathways to the airway wall



myelinated slow adapting 'stretch' receptors, rapidly adapting 'irritant' receptors, and non-myelinated C-fibre endings [see Coleridge and Coleridge 1984, Widdicome 1986]. Nerve terminals resembling C-fibre endings have been identified in human airway epithelium [Laitenen 1985a, Rhodin 1966]. Little is known, however, of the distribution of C-fibre endings in deep lung, where they are thought to represent the 'J'-receptors (juxta-capillary receptors) which are activated by lung deflation [Coleridge and Coleridge 1984], Fox and colleagues [1980] have suggested that the alveolar nerves observed in their study might represent these endings. C-fibres are thought to contain substance P, which is released in response to capsaicin [Lundberg and Saria 1983]. Inhalation of capsaicin in humans causes cough and rapid, transient bronchoconstriction, indicating stimulation of laryngeal C-fibre endings [Fuller et al 1985]. However, because of the cough response, which prevents capsaicin from reaching the smaller airways, it is not possible to study the effects in peripheral lung. The sparcity of substance P and CGRP found in the present study suggests that they may not be involved in senory responses in peripheral lung. However, resolution at light microscopic level may not be sufficient to identify individual nerve endings. Therefore, the existence of afferent nerve endings in peripheral lung containing substance P and CGRP should not be discounted.

Previously, there have been no quantitative, histochemical studies on the density of neural markers at different levels in the human lung. The finding of the present study of a general sparcity of cholinergic nerves in peripheral lung, but no significant decreases at the level of the bronchioles in aminergic or VIPergic innervation, raises questions about the role of inhibitory innervation in the smooth muscle of smaller airways. In chronic airway obstruction, the principle site of resistance to airflow is located in small airways of a diameter of less than 2mm [Hogg et al 1968]. In the present

study, airways of this size were located mainly at airway level IV.

Observations of the density of acetylcholinesterase in the present study, indicated that the density of cholinergic nerves significantly decreased in bronchial smooth muscle with increasing airway level. This finding is supported by the observation of a decreased number of muscarinic receptors in human peripheral lung [Raaijmakers et al 1984]. However, Finney et al have demonstrated atropine-sensitive contraction of bronchiolar smooth muscle in response to carbachol in a preparation of human small airways (0.6 - 1.5mm diameter) [Finney et al 1985] indicating that there may be some functional cholinergic innervation in peripheral airways.

In the present study, VIPergic nerves at different airway levels showed no significant decrease in the innervation of bronchial smooth muscle up to airway level IV. This finding contrasts with that of other workers who have reported a sparcity of VIP in bronchioles [Dey et al 1981, Laitenen et al 1985a]. Also, VIP has been shown to relax human bronchi, but not bronchioles in vitro [Palmer et al 1986]. VIP receptors are found in greater density in the bronchi than the bronchioles of human airways [Palmer et al 1986, Carstairs and Barnes 1986b]. It is therefore possible that the reduced response of smaller airways to VIP is due to a reduction in receptor density rather than a reduction in the density of nerves.

The present study has also shown that the incidence of aminergic nerves in bronchial smooth muscle does not decrease with increasing airway generation. In support of this observation, relaxant effects of noradrenaline have been reported in bronchioles [Finney 1985]. However, the latter observation was qualitative and was not examined in the presence of carbachol. Relaxation of human bronchioles in response to isoproterenol has been reported [Palmer et al 1986]. Neural effects of noradrenaline are mediated by β_1-receptors

[Ariens and Simonis 1983] and other workers have been unable to demonstrate β-1 receptor mediated relaxation of human bronchial smooth muscle [Zaagsma et al 1983]. The relaxant effect of isoproterenol in peripheral airways [Palmer et al 1986], therefore, is probably mediated by β_2-receptors.

In the present study, the submucosal gland did not show any significant decrease in cholinergic, aminergic or VIPergic nerves. Submucosal gland was generally only present to airway level III (i.e. segmental airways). The findings of the present study would suggest that there is no functional difference in control of mucus secretion in smaller airways. However, there is at present no functional evidence to confirm or deny this assumption.

4.2. THE DISTRIBUTION OF NEURAL MARKERS IN DISEASE.

In this section, the findings in relation to cystic fibrosis and asthma are discussed and the functional implications of the observed changes are considered.

4.2.1. Cystic fibrosis.

The exocrinopathy of CF is characterised by disorders of the control mechanisms of secretion. Most exocrine glands, including the bronchial submucosal glands, are under cholinergic, adrenergic [Sato 1977] and peptidergic control [Holkfelt et al 1980]. Autonomic defects are characteristic of CF (see Introduction, section 2.1). In the lung, for example, secretion of mucus is abnormally increased in response to cholinergic agonists [Sturgess and Reid 1972a].

Overall, evidence indicates that patients with CF have increased cholinergic and alpha-1 receptor-mediated sensitivity in conjunction with reduced beta-2 adrenergic responses. Parents of CF patients show many of these abnormalities to a lesser degree indicating the primary nature of the defect [Davis and Kaliner 1983].

In the present study, direct comparison of lung tissue taken from CF patients with that from 'normal' patients has revealed that there is no gross abnormality in the innervation of the lung. The distribution of PGP 9.5 to effector structures is not grossly altered, although a slight, but statistically significant ($p<0.05$) reduction in the numbers of nerves in the epithelium was noted (The implications of this are discussed in section 2.2.)

Comparison of normal and cystic fibrosis airways revealed no differences in

the distribution of acetylcholinesterase at any airway level in any of the effector structures examined. Observations on the density of aminergic nerves (as delineated by tyrosine hydroxylase) and NPY have revealed no marked difference between CF and normal airways, assessed in a semi-quantitative fashion. Similarly, examination of the 'sensory' component has revealed no differences in the distribution of substance P or CGRP.

The major difference observed in the present study, was apparent in the VIP-ergic supply. This was most marked in the submucosal glands where there was a significant reduction in the VIPergic innervation ($p<0.001$) compared with 'normal' tissue. Bronchial smooth muscle and ganglia were also affected, but to a lesser extent than the submucosal gland. The difference was not attributable to a post-mortem artifact (4 out of 5 cases of CF were freshly-fixed transplant tissue) and the difference was observed in both the benzoquinone-fixed tissue and the paraffin-embedded samples.

VIP has been shown in physiological studies to have potent effects on blood flow [Malm et al 1980], macromolecular secretion [see Nadel 1985] and the transport of chloride ions and water across intestinal and tracheobronchial mucosa [Krejs et al 1980, Nathanson et al 1983]. VIP also reduces the potential difference across the duct of submucosal gland [Dennis and Young 1978]. These observations suggest that VIP normally exerts a physiological regulatory influence on exocrine gland function and that the observed defects in CF could be accounted for by a paucity of VIP. In the gut, an absence or reduction in VIPergic innervation has been implicated in the pathophysiology of Hirchsprung's disease and oesophageal aschalasia. The following observations indicate that an abnormality in the VIPergic supply may well exist in CF:-

i. In the sweat glands from patients with CF, a reduction in VIP immunoreactivity in association with both the acini and duct portions of

the gland has been reported [Heinz-Erian et al 1985].

ii. In jejunal biopsies taken from CF patients there is an increase in the number of VIP-immunoreactive cells that is directly correlated to the degree of abnormal mucus secretion and severity of steatorrhoea. These cells are in direct association with the intestinal goblet cells [Buchanan et al 1983].

However, in the present study, the possibility that the observed reduction in VIPergic innervation in the lung is of primary importance in the pathology of CF is unlikely in view of the observation that both the bronchiectatic and 'young' control tissue also showed a deficiency of VIP in the submucosal gland compared to the normal tissue. However, the distribution of PGP 9.5 was unchanged in all groups. This suggests that damage has not occured to the nerves and ganglia themselves, but to the neurotransmitters they contain. A reduction in the levels of VIP may come about secondary to infection of the lung: proteolytic enzymes produced by bacteria and the inflammatory response could cause the breakdown of VIP. In the present study, an indication that more widespread damage to VIP may occur in disease, was apparent in the reduction in density observed in the ganglia and the bronchial smooth muscle, of the CF, bronchiectatic and 'young' tissue compared with the normal tissue. This observation is supported by the finding of a reduced number of VIP receptors in CF airways in comparison with 'normal' airways [R. Sharma, unpublished observations].

Direct comparison of the CF tissue with the 'young' tissue showed a significant reduction of VIP in the gland ($p<0.05$), but not in the bronchial smooth muscle or ganglia. Further analysis of the results has revealed that the density of VIP is inversely related to the area of submucosal gland and the correlation is statistically significant ($p<0.05$). Also, radioimmunoassay revealed no significant difference between the 'normal' controls and the CF

tissue in the level of VIP. Therefore, assuming the total amount of VIP in the lung remains the same in disease, then the effect of mucous gland hypertrophy would be to dilute the observed density of nerve fibres, resulting in the observer awarding a lower score during the semi-quantitative assessment. The effect of this 'thinning out' of the nerve fibres may result in a reduced control by VIP of the secretory processes. Evidence for this has been provided in a study by Coles [Coles et al 1981] who has demonstrated that in normal lung, cholinergically induced mucus secretion (as measured by glycoprotein output) is inhibited by VIP. However, in chronic bronchitis, where there is mucous gland hypertrophy, the inhibitory effect of VIP is less marked.

Observations of the density of catecholamines, NPY, substance P and CGRP in bronchiectatic tissue have also revealed a reduction in density in comparison to the normal, 'young', asthmatic and cystic fibrosis tissue. Subjective observation of the bronchiectatic tissue showed that it was distinguished from the other groups of tissue in that the samples showed evidence of more extensive inflammation and mucus-hypersecretion. Therefore, in the bronchiecatatic tissue, the generalised reduction in neural markers may have been partly due to the extensive inflammatory damage. Other workers have shown that inflammatory mediators reduce the number of β-receptors in the lung [Raaijmakers et al 1986], although it is not known whether the same mediators damage the nerve supply. The results of the present study indicate that reduced levels of neurotransmitters are secondary to infection and inflammation in disease. Such a reduction may lead to abnormal control of effector structures in the lung, and hence contribute to the symptoms of chronic obsructive lung disease.

4.2.2. Asthma.

Many of the theories put forward to explain the bronchial hyperreactivity characteristic of asthma are based on the idea of autonomic abnormality. Until recently, theories have centred either on hyperresponsiveness of cholinergic excitation of bronchial smooth muscle, or an underactivity of the adrenergic inhibitory system. Evidence for and against these hypotheses has been presented in various reviews [see Simonsson et al 1967, Szentivanyi 1968, Reed 1974]. These theories are now being modified to include the possible involvement of the recently discovered NANC system. An enhanced non-cholinergic excitatory response or reduced non-adrenergic response could contribute to the symptoms of bronchial hyperreactivity [see Richardson 1981, Lundberg et al 1983, Barnes 1986c, Barnes 1987].

Studies on asthma have been limited by the availability of fresh tissue; in the present study most of the information is based on retrograde studies of formalin-fixed, paraffin-embedded tissue from patients dying in status asthmaticus. In addition, evidence has been obtained from tissue from one patient, resected for carcinoma, but with a history of asthma. Observations of the density of all types of nerves in this case revealed no obvious anatomic abnormality in any of the neural components supplying the lung.

4.2.2.1. The epithelium in asthma.

Epithelial damage and sloughing is a feature characteristic of even mild asthma and it appears to correlate with the degree of bronchial hyperresponsiveness [Laitenen et al 1985b, Jeffery et al 1987]. Damage to intra-epithelial nerves and consequent regrowth may contribute to hyperreactive airways: nerve growth factor (NGF) can cause more than a

100% increase in histamine release from mast cells when the cells are preincubated with NGF and subsequently exposed to antigen [Comsa et al 1982]. NGF has also been shown to cause a relatively selective increase in mast cell growth in cultured mast cell from human blood mast cells [Staniz et al 1987a]. However, in the present study, examination of asthmatic tissue revealed no change in the number of nerves within the epithelium (compared with the 'normal' group), or nerves exposed to the airway lumen in areas of epithelial shedding. In the CF patients, a slight, but statistically significant ($p<0.05$) reduction in the number of intra-epithelial nerves was observed in comparison with the normal and the 'young' tissue. However, there was no difference between CF and asthmatic or bronchiectatic tissue. The apparent reduction in intra-epithelial nerves may be explained by the dilation of airways that occurs due to chronic mucus plugging in CF, bronchiectasis and asthma. A consequent reduction in density of nerves in dilated airways compared to 'normal' airways would be expected, assuming their total number were to remain unchanged.

In the present study, hyperplasia of substance P and CGRP-immunoreactive nerves in the epithelium and lamina propria was observed in one asthmatic and one normal case. Hyperplasia of nerves stained with PGP 9.5 in the lamina propria was observed in 2 out of the 6 cases of asthma and in one out of the 7 normal cases examined in this study. The overall distribution of substance P and CGRP to all effector structures in the lung showed no difference between 'normal', asthmatic, CF and 'young' tissue, again suggesting that there is no gross abnormality in the distribution of sensory neurotransmitters specific to asthma. Neither was there any apparent change in the number of ganglia observed in asthmatic airways.

A hypothetical increase in airway afferent receptor discharge has been put forward to explain the airway hyperreactivity observed in asthma.[Simonsson et al 1967, Barnes 1986]. Increased release of substance P could account for many of the symptoms of asthma, including increased bronchial smooth muscle contraction and airway oedema [Karlsson et al 19874, Lundberg et al 1983c]. CGRP may also be implicated in asthma, especially if it is co-released with substance P, as it is a potent bronchoconstrictor and vasodilator [Palmer et al 1987, Lundberg et al 1985a].

Increased afferent activity might occur due to inflammation and the consequent release of histamine, leukotrines and prostaglandins [Fuller et al 1986, Fuller et al 1987] or by the exposure of afferent nerve endings below and within the epithelium during epithelial sloughing, but there is no evidence from the present study to support this idea. However, damage to the airway epithelium in asthma could contribute to the hyperreactive state by increasing airway permeability to inhaled irritants, which may then act directly on the 'target' cells (e.g. sensory nerves, inflammatory cells or bronchial smooth muscle). For example, removal of the epithelium of in vitro preparations of human airway has been shown to increase the contractile effect of methacholine [Raeburn et al 1986].

There is evidence that the increase in airway reponsiveness after inhalation of allergens or ozone in humans is associated with an increase in the number of inflammatory cells [Stelzer et al 1986, De Monchy et al 1985]. In the absence of evidence from the present study of increased numbers of sensory nerves in asthma, it is possible that the existing nerves interact with increased numbers of inflammatory cells. Depletion of sensory neuropeptides in animals by capsaicin pretreatment reduces airway responses to inflammatory mediators [Lundberg and Saria 1983]. It has been postulated that substance P might amplify neutrophil and eosinophil responses to

chemotactic agents and thereby magnify the acute inflammatory response in the airways [Payan et al 1984]. Substance P receptors have also been demonstrated on both T and B lymphocytes [Stanisz et al 1987b].

There is substantial evidence that axon reflexes involve mast cells and the interaction may be mediated by substance P-containing nerves [Kiernan 1975, Erjavec et al 1981, Piotrowski and Jordan 1982]. Intestinal mucosal mast cells have been shown to secrete in response to substance P [Stanisz et al 1987a]. Although the presence of nerves in association with mast cells has not been demonstrated in the present study, there is evidence from other work that mast cells may be 'innervated' by nerves fibres, possibly containing substance P [Wiesner-Menzel et al 1981, Skofitsch et al 1985]. Human airway ganglia have been shown by other workers to contain mast cells [Jeffery 1982]. As substance P nerves have been shown to be located within human airway ganglia the possibility exists for an indirect effect of substance P on neural transmission via the release of inflammatory mediators from these mast cells, which may influence ganglionic transmission. It has been shown that pretreatment of atopic subjects with an aerosol of hexamethonium (which blocks ganglionic transmission) reduces the exaggerated response to inhalation of histamine, but not to methacholine [Holtzman et al 1980]. This suggests that the response to histamine is neurally mediated, whereas methacholine acts directly on the muscarinic receptors of the smooth muscle to cause bronchoconstriction.

In the absence of evidence for an increased incidence of the 'sensory' neurotransmitters (substance P and CGRP) in asthmatic airways, it seems unlikely that this is a direct cause of bronchial hyperreactivity and airway oedema. However, the possibility exists of an involvement in the release of mediators from mast cells (substance P) and in neuromodulation of transmission through parasympathetic ganglia (substance P and CGRP).

4.2.2.2. Bronchial smooth muscle in asthma.

a. Cholinergic mechanisms. Cholinergic nerves are the dominant bronchoconstrictor neural pathway in man [Nadel 1980], therefore it is logical to consider that an overactivity of the cholinergic system would lead to the bronchial hyperreactivity characteristic of asthma. This assumption is supported by the fact that many stimulants of bronchospasm in asthmatics also stimulate vagal afferents [Nadel et al 1965]. Asthmatics also show an abnormally increased sweat production and pupillary dilation in response to cholinergic agonists [Kaliner et al 1982]. In acute, severe asthmatic attacks, anti-cholinergic drugs relieve bronchoconstriction, suggesting reflex vagal involvement [Ward et al 1981]. Yet anti-cholinergic drugs are not generally clinically effective in chronic allergic asthma [Mann and George 1985], suggesting the involvement of other mediators. One of the mechanisms that could account for the increased activity of bronchial smooth muscle is a defect in the efficacy of acetylcholinesterase, which could lead to increased amounts of acetylcholine and hence increased bronchial smooth muscle contraction. Asthmatics show a responsiveness to inhaled cholinesterase inhibitors which is greater than normals and is related to the degree of sensitvity to methacholine [Quigley et al 1985] and which could be due to increased responsiveness of the airway smooth muscle to endogenous acetylcholine. In vitro studies of asthmatic airways, obtained at surgery, fail to demonstrate the same increases in cholinergic responsiveness in the bronchial smooth muscle as seen in vivo [Roberts et al 1984]. This implies that there is no inherent abnormality in the airway smooth muscle but in its neurohumoral control. However, cholinesterase, measured in whole blood, has been found to be normal in asthma, even during an attack [Hawes and Alles 1940]. Neither is there a correlation between the severity of asthma and

the blood level of acetylcholine in asthmatics However, the mean level of acetylcholine in the blood of asthmatics has been reported to be significantly higher than in the blood of normal patients (although there is considerable overlap between the two groups) [Scudamore 1954]. Large quantities of acetylcholine have also been found in the blood of rabbits in anaphylactic shock [Wenner and Buhrmester 1937]. In view of the finding of these workers that acetylcholinesterase activity appears unimpaired, butyryl-cholinesterase (BuchE) was investigated in the present study. A reduction in the levels of BuchE in canine serum has been associated with an increase in pancreatic ductal tension [Dressel et al 1980] and in the cat, inhibition of BuchE causes an increase in spontaneous contractile activity of the ileum [Koelle 1950]. It is therefore possible that reduced BuchE in the airways of asthmatics could contribute to a hyperreactive state. However, in the present study the levels of BuchE in asthmatic serum were found to be no different to normal, or to serum from asthmatic or hyperreactive patients. Obviously, levels in the serum may not reflect local changes in the effector structure in the lung, however in the one case of fresh asthmatic tissue available, the histological distribution of BuchE was unaltered.

b. Adrenergic abnormality in asthma. Compared with normal tissue, there was no difference observed in the density of aminergic innervation in the one available case of fresh asthmatic tissue. The findings of a sparse sympathetic innervation of normal airways in bronchial smooth muscle, coupled with the presence of large numbers of beta-receptors [Zaagsma et al 1983, Carstairs et al 1985] has raised questions about the role of the sympathetic inhibitory innervation of the smooth muscle in asthma. It has been suggested that the β_2-receptors on the smooth muscle make it susceptible to influence by circulating catecholamines and this interaction

may somehow be defective in asthma [Barnes 1986]. However, plasma concentrations of catecholamines in asthma are no different to those in plasma from control subjects, nor is there any correlation between the degree of bronchoconstriction and plasma catecholamines as judged by % predicted lung function [Barnes et al 1982]. Circulating catecholamines may, however, have a role in influencing mast cell degranulation via β_2-receptors. In the guinea-pig, B-agonists are more effective in allergen induced bronchospasm in vitro [Adams et al 1984, Assem and Schild 1969] and noradrenaline shows inhibitory effects against IgE- induced histamine release in human lung fragments [Butchers et al 1980]. Exercise causes a rise in circulating catecholamines in normal subjects but not in asthmatics. Even though they bronchoconstrict there is only a limited rise in plasma noradrenaline and no change in the levels of adrenaline [Barnes 1986b, Warren and Dalton 1983]. However, asthmatics are often able to 'run through' their asthma [Larsson et al 1982] suggesting a defect in mobilisation of catecholamines. Even in acute asthma, there is no increase in the levels of circulating adrenaline but noradrenaline is increased. The latter observations, however, may be a reflection of increased respiratory muscle activity [Ind et al 1985]. Under other circumstances, asthmatics secrete adrenaline normally [e.g. insulin induced hypoglycaemia, [Ind et al 1983]. β-blockers or adrenalectomy fail to turn normal people asthmatic, therefore it is unlikely that reduced production of catecholamines is a primary defect in asthma. Similarly a defect in β-receptor function is unlikely for the same reason, although there is much debate about a possible defect in the secondary messenger mechanism [Barnes et al 1984, Barnes 1985]. These observations suggest that a defect in the noradrenergic control of the airways is not in itself the underlying cause of bronchial hyperreactivity. However, the observations of the present study, of catecholamines in airway

ganglia and bronchial smooth muscle, indicate the possiblity for an indirect, neuromodulatory role for noradrenaline in the control of airway smooth muscle. The functional existence of such an effect and the consequences of a possible dysfunction in asthma, however, remain hypothetical.

c. NANC mechanisms. Maximum muscarinic blockade does not result in complete inhibition of the broncoconstrictor response. Patients with asthma continue to exhibit abnormally large bronchoconstrictor responses to inhaled irritants after treatment with cholinergic antagonists, which suggests that the stimulus acts partly by parasympathetic mechanisms and partly by other mechanisms [Tam et al 1983]. The apparent sparsity of aminergic inhibitory innervation to bronchial smooth muscle shown herein and in other studies, has raised questions of the role of the NANC system in asthma. In the present study, VIP has been demonstrated in abundance in bronchial smooth muscle down to the level of the segmental bronchi. Its role in asthma as yet remains theoretical. In bovine tracheal muscle, VIP has an inhibitory effect on cholinergic nerve-induced contraction, but only at high frequency firing [Palmer et al 1985]. This suggests that VIP may act as a 'braking' mechanism on cholinergic contraction [Barnes 1987]. In asthma, mediators released from mast cells, neutrophils and eosinophils could breakdown VIP which is susceptible to enzymatic attack. This would block the relaxation response induced by VIP leading to increased cholinergic tone [Barnes 1986]. However, studies on inhaled VIP have shown that VIP has only a slight protective effect on histamine-induced bronchoconstriction [Barnes and Dixon 1984] possibly due to VIP being broken down before it reaches the bronchial smooth muscle. Although the present study revealed no difference in the VIPergic supply in tissue obtained at resection from one patient with a history of asthma, it is not clear whether the levels of VIP are reduced in

more severe asthma. The observation herein, of a reduction in the density of VIP in the bronchial smooth muscle of CF tissue, may partly explain the hyperreactivity evident in the case history of some of these patients [Mitchell et al 1978].

The role of NPY in asthma has still to be investigated but in view of its action in inhibiting cholinergically induced smooth muscle contraction [Stretton and Barnes 1988] it is potentially of importance in asthma.

In view of the findings in the present study of negligible amounts of substance P and CGRP in the smooth muscle of both normal and asthmatic airways it seems unlikely that these peptides are directly involved in causing increased smooth muscle contraction directly, but may act through the airway ganglia, or by effects of their local release in the epithelium or by affecting mast cells.

4.2.2.3. The submucosal glands in asthma.

Post-mortem examination of the fatal cases of status asthmaticus has revealed diffuse secretions of mucus which appear to contribute significantly to the airways obstruction [Messer et al 1960]. VIP has been shown to inhibit antigen-induced histamine release from guinea-pig lung homogenates [Undem et al 1983] and therefore a reduction of VIP, which may occur in severe asthma due to breakdown by proteolytic mediators, may lead to an increased production of histamine. Anaphylactic release of histamine and slow-reacting substance of anaphylaxis (SRS-A) has been shown to increase glycoprotein secretion via stimulation of histamine receptors [Shelhamer er al 1980]. A reduction in the levels of VIP may lead directly to an increased mucus output as VIP has been shown to inhibit cholinergically stimulated mucus production in human airway in vitro [Coles et al 1981]. It is not known whether VIP directly inhibits histamine-induced mucus secretion.

However, other mediators of anaphylaxis have a much more potent effect on mucus secretion than histamine, notably leukotrines and prostaglandins [Shelhamer et al 1982].

Increased substance P activity could also lead to mucus hypersecretion as it stimulates mucus secretion from canine and human trachea [Coles et al 1984, Barnes et al 1986]. However the effect in man is unlikely to be direct as the present study has shown that substance P is not present in the submucosal gland and does not appear to be abnormally increased in asthma. However, substance P may act to increase mucus secretion indirectly via the release of inflammatory mediators from mast cells [Staniz et al 1987]. Therefore, mucus hypersecretion in asthma probably occurs as result of the release of inflammatory mediators, which may be influenced by both VIP and substance P.

4.3. FUTURE DIRECTIONS.

The results and discussion of the studies presented herein indicate a number of directions for future research either in the form of new work or to clarify existing controversy.

1. The role of VIP in the pathogenesis of mucus hypersecretion.

The results of the present study suggest a speculative link between the degree of mucous gland hypersecretion and the apparent sparcity of VIPergic innervation. Mucous gland hypertrophy is a feature characteristic of airway hypersecretory diseases such as chronic bronchitis, asthma and cystic fibrosis. Physiological studies should be carried out on available tissue transplanted from patients with cystic fibrosis and 'normal' or disease control tissue, to investigate further the role of VIP in the control of mucous gland secretion. Mucus secretion in response to VIP from human airways in vitro could be assessed using biochemical assays for mucus markers (e.g. fucose) to determine the amount of mucus produced. Mucus synthesis could be measured by the use of radiolabelled precursors of mucus. These experiments could be supplemented with ultrastructural studies on the tissue after exposure to VIP to determine the extent of mucous and serous cell degranulation, and immunohistochemical studies to determine the extent of VIPergic innervation in the airways used for experimentation.

There is evidence that neural receptors may be damaged in disease; some inflammatory mediators may be involved in causing damage to β-receptors in the lung [Raaijmakers et al 1986]. Studies could be carried out on available human lung tissue to determine the extent of damage to the VIP receptors in

disease. For example, autoradiography could be carried out to determine the number and binding affinity of the receptors on the submucosal glands, and also functional studies should be done on the ability of VIP to stimulate cAMP formation in normal and diseased lung.

2. The role of peptides and catecholamines found in human airway parasympathetic ganglia.

The findings of several distinct neurotransmitters in airway ganglia has important implications for the neural control of the airways. The degree of presynaptic modualation that may occur and the pathological implications of a dysfunction are of relevance to understanding both normal control and changes in disease. There is potential for a direct role of neuropeptides in the modulation of ganglionic transmission and an indirect role, via the release of inflammatory mediators from mast cells within the ganglia. Therefore, physiological studies should be done on the role of these transmitters in the parasympathetic ganglia using the currently available supply of fresh human tissue from resection procedures and the transplantation program. Physiological studies could be supplemented with ultrastructural and immunocytochemical techniques to help distingush the exact location of the different neurotransmitters within the ganglia, especially in relation to mast cells.

3. The role of Butyrylcholinesterase.

The present study has demonstrated the presence, in human airway vascular and bronchial smooth muscle, of relatively large amounts of butyryl-cholinesterase, the role of which is unknown. The results of studies done by

other workers indicate that it may be of physiological importance and therefore its role should be investigated further. In vitro, physiological studies of the effect of the specific inhibition of butyrylcholinesterase on human airway bronchial smooth muscle should be done. Biochemical investigations of the peptidase activity of butyrylcholinesterase should also be carried out to attempt to clarify its potential physiological role and its effects on neuropeptides in human lung.

4. The role of the symapthetic component in human bronchial smooth muscle.

The results of this study and those of other workers have indicated a sparse sympathetic supply to human airway smooth muscle. In particular, the present study has demonstrated the presence of NPY in human airway smooth muscle, which could be localised in sympathetic nerves. Therefore functional studies should be carried out on the role of the sympathetic supply to human airway smooth muscle, using the available supplies of human airways. Such studies could include experiments similar to those carried out on guinea-pig airways [Stretton and Barnes 1988], to determine the effect of NPY on cholinergic nerve-mediated bronchial smooth muscle contraction in man.

5. The role of the autonomic nervous system in the control of airway vasculature.

The lung is the only organ in the body through which the entire blood supply must pass. The results of this study and those of other workers have shown that both bronchial and pulmonary vasculature recieves a rich supply of aminergic and peptidergic nerves. Both sympathetic and NANC transmitters have been shown to have profound effects on human systemic vasculature in

other studies [Laitenen et al 1987a]. It has been suggested that the contractions in response to noradrenaline observed in strips of peripheral lung are due to the response of vascular smooth muscle rather than airway smooth muscle, which is more sparse in peripheral lung [Finney et al 1985]. It is suggested, therefore, that in vitro functional studies on strips of human peripheral lung could provide information about the response of vascular smooth muscle to exogenously applied putative neurotransmitters in the presence of either sympathetic or parasympathetic antagonists. By labelling the antagonists applied to the lung tissue e.g. with radioactive isotopes, subsequent autoradiographical examination could help to distinguish whether the observed effects are due to interaction of the antagonist with the vascular or bronchial smooth muscle.

6. Controls and precautions for future investigations.

The difficulty of histological studies, especially those concerned with retrograde studies on fixed and embedded tissue, is in determining whether an observed sparsity is due to an inherent sparsity or a reduction due to tissue processing, age of the patient etc. Therefore any future study in addition to the usual controls, should be supplemented with:-

i. Radioimmuno- or biochemical assay of the substance in the tissue sample.

ii. The use of tissue from young patients where possible to ensure that alterations in the levels of any substance of interest is not age-related.

iii. Post-mortem tissue should only be used in the circumstance of a lack of any other available tissue source and any inferences drawn from

such material should be made with caution.

iv. Control tissue from animals known to be positive for the substance under investigation should be used at all times.

The facilities for making all the above determinations are presently available. Human tissue samples are readily available from resection and the increased implementation of a lung transplantation program. The latter is providing an alternative supply of tissue, usually from younger patients with a variety of lung diseases rather than the tissue from resections which tends to come from older patients with carcinoma and a history of smoking.

It should also be noted for any future physiological investigation that the findings of this study and those of other workers have indicated that there are important species differences in the extent and type of innervation in the lung and therefore care should be taken when extrapolating the results of studies done on animals to man.

The findings in this study of a general reduction in the nerve supply to the airways when the tissue has been severely damaged by disease warrant further investigation. It is not yet clear to what extent damage to the nerve supply may contribute to the manifestations of chronic lung disease, but clearly there is ground for further study.

4.4. GENERAL SUMMARY.

1. The function of the human lung is controlled by the autonomic nervous system which has parasympathetic, sympathetic and NANC components. Autonomic abnormality has been implicated in several disorders of man including asthma and cystic fibrosis of the lung.

2. Histochemical and immunohistochemical techniques are used to delineate the components of the autonomic nervous system in 'normal', cystic fibrosis and asthmatic lung. Control tissue was also taken from bronchiectatic lungs and from a group of young patients (average age 28).

3. The overall supply of nerves shows a general reduction in the density of neural markers with increasing airway generation. No neural marker was found beyond the level of the subsegmental bronchi.

i. Substance P and CGRP are generally sparse in human airways but form a significant nerve supply to the airway lining epithelium and are consistently present in airway ganglia. CGRP is present in neuroendocrine cells.

ii. Cholinergic nerves as delineated by acetylcholinesterase histochemistry are present in bronchial smooth muscle, submucosal gland and the lamina propria, but not the lining epithelium of the airways. A sparse supply of cholinergic nerves was present around bronchial and small pulmonary vessels. Ganglia in the airways are all strongly positive for cholinesterase. Butyrylcholinesterase is present diffusely in bronchial and vascular smooth muscle and the density of stain increases with increasing airway generation.

iii. VIP shows a similar distribution to the cholinergic supply with a dense supply of nerves to the bronchial smooth muscle, submucosal gland and lamina propria. It is also present in high density in the airway ganglia. In contrast to the parasympathetic supply, VIP is consistently present around both bronchial and pulmonary vessels to the level of the segmental airways.

iv. Aminergic nerves, as delineated by glyoxylic acid-induced fluorescence and immunohistochemistry for tyrosine hydroxylase, are generally more sparse than the cholinergic supply. An exception to this is in the bronchial and pulmonary vessels, which are densely innervated. Aminergic nerves are also present in submucosal gland and, to a lesser extent, bronchial smooth muscle and airway parasympathetic ganglia down to the level of the sub-segmental airways.

v. NPY shows a similar distribution to the aminergic supply to the airway vasculature and ganglia. However, NPY is not present in submucosal gland but is consistently present in bronchial smooth muscle.

4. The bronchiectatic tissue shows a reduction in density of all the neural markers under investigation. This reduction was concluded to be a result of the extensive tissue damage apparent in these tissue samples.

5. In cystic fibrosis the innervation appears normal except for a reduction in the density of VIP in the submucosal gland and airway ganglia. This reduction appears to be related to the degree of mucous gland hypertrophy.

6. There are no obvious differences in the autonomic nerve supply to the lung in asthma. In particular, there is no difference in the area of tissue occupied by the airway ganglia and no differences in the epithelial innervation or distribution of substance P in comparison with the normal tissue. The presence of a sympathetic component in the bronchial smooth muscle and airway ganglia and the presence of neuropeptides in the ganglia have implications for the control of the airways and the consequences, in terms of bronchial hyperreactivity, of a possible dysfunction.

APPENDICES.

APPENDIX 1.

Tables of patient details.

1. 'Normal' patients

No.	M/F	Age	D	C	S	R.T	B	St.	H	R	T
149	M	70	Ca.	x	/	/	x	x	x	R	<½hr
155	M	61	Ca.	x	/	/	x	x	x	R	<½hr
152	M	73	Ca.	x	/	x	x	x	x	R	<½hr
154	M	65	Ca.	H.T	/	x	x	x	x	R	<½hr
156	M	64	Ca.	x	/	x	x	x	x	R	<½hr
77	M	70	Ca.	x	/	x	x	x	x	R	2hrs
85	M	68	Ca.	x	/	x	x	x	x	R	<½hr
86	M	65	Ca.	x	/	x	x	x	x	R	2hrs
87	M	51	Ca.	x	/	/	x	x	x	R	<½hr
122	F	69	Ca.	E	/	x	x	x	x	R	<½hr
80	F	54	Ca.	x	/	/	x	x	x	R	<½hr
40	M	57	Ca.	x	/	x	x	x	x	R	<½hr
136	M	71	Ca.	x	/	x	x	x	x	R	1hr
186	F	54	Ca.	x	/	x	x	x	x	R	<½hr
167	M	64	Ca.	x	/	x	x	x	x	R	<½hr
168	M	45	Ca.	x	/	x	x	x	x	R	<½hr
230	F	70	Ca.	x	/	x	x	x	x	R	<½hr
262	F	46	Ca.	x	/	x	x	x	x	R	<½hr
332	F	62	Ca.	x	/	x	x	x	x	R	<½hr
236	M	70	Ca.	x	/	x	x	x	x	R	<½hr
336	F	58	Ca.	x	/	x	/	x	x	R	1hr
302	M	48	Ca.	x	/	x	x	x	x	R	<½hr
365	F	65	Ca.	A	x	x	x	x	x	R	<½hr
353	M	68	Ca.	x	/	x	/	x	x	R	<½hr
329	F	57	Ca.	x	/	x	x	x	x	R	<½hr
200	F	27	Ho.	x	x	/	x	/	x	R	<½hr
204	F	62	Ca.	x	/	x	x	x	x	R	<½hr
184	M	47	Ca.	E	/	x	x	x	x	R	<½hr
206	M	78	Ca.	x	/	x	x	x	x	R	<½hr
189	F	58	Ca.	x	/	x	x	x	x	R	<½hr
205	M	59	Ca.	x	/	x	x	/	x	R	<½hr

2. Young Patients.

No.	M/F	Age	D	C	S	R.T	B	St.	H	R	T
217	F	3	Cy.	B	x	x	/	/	/	R	<½hr
333	M	62	Ca.	B	/	x	/	/	/	R	<½hr
218	M	16	Sa.	x	x	/	x	x	x	R	<½hr
216	M	17	Cy.	x	/	x	x	x	x	R	<½hr
229	M	37	Sa.	x	/	x	x	x	x	R	<½hr
287	F	32	Ca.	B	/	x	/	x	x	R	<½hr
215	F	36	Ca.	x	x	x	x	x	x	R	<½hr
277	F	18	E	x	x	x	x	x	x	x	<½hr

3. Bronchiectatic Patients.

No.	M/F	Age	D	C	S	R.T	B	S	H	R	T
740	F	42	B/E	My.	x	x	x	x	x	R	<½hr
236	M	12	B/E	x	x	x	x	x	x	R	<½hr
233	M	9	B/E	x	x	x	x	x	x	R	<½hr
415	F	43	B/E	x	x	x	x	x	x	R	<½hr
542	F	40	B/E	Eo.	x	x	x	x	x	R	<½hr
340	F	38	B/E	x	x	x	/	x	x	R	<½hr
500	F	67	B/E	H.T	x	x	x	x	x	R	<½hr

4. Cystic Fibrosis.

No.	M/F	Age	D	C	S	R.T	B	St.	H	R	T
322	M	32	CF	x	x	x	/	/	/	PM	3hrs
318	F	3	CF	x	x	x	x	x	x	R	<½hr
327	F	30	CF	x	x	x	/	x	x	T	<½hr
321	M	37	CF	C.P	x	x	/	x	/	T	<½hr
334	F	29	CF	As.	x	x	/	/	/	T	<½hr
316	F	23	CF	x	x	x	/	x	/	T	<½hr
211	F	25	CF	x	x	x	/	/	/	T	<½hr
283	M	18	CF	Ar.	x	x	/	/	/	PM	2hrs
358	F	23	CF	Db.	x	x	/	/	/	T	<½
222	M	18	CF	x	x	x	/	/	/	T	<½
175	F	20	CF	x	x	x	/	/	/	PM	5hrs
172	F	29	CF	Db	x	x	/	/	/	PM	12hrs

5. Asthma.

No.	M/F	Age	D	C	S	R.T	B	St.	H	R	T
313	M	61	Ca.	As.	/	x	/	x	x	R	<½
5/7	M	63	As.	/	/	x	/	/	/	PM	24hrs
28	M	13	As.	/	/	x	/	x	/	PM	48hrs
19	F	64	As.	/	/	x	/	/	/	PM	20hrs
256	M	36	As.	/	/	x	/	/	/	PM	40hrs
5/2	F	39	As.	/	/	x	/	/	/	PM	18hrs
2	F	16	As.	/	/	x	/	/	/	PM	72hrs
13	M	25	As.	/	/	x	/	/	/	PM	72hrs
10	M	42	As.	/	/	x	/	/	/	PM	72hrs

Codes.

No. = Patient code number.

M/F = Male/Female.

D = Major diagnosis: Ca.=cancer, Sa.=sarcoma, E=emphysema, Cy.=lung cysts, Ho.=Hodgkin's, As.= Status asthmaticus, B/E=bronchiectasis, CF=Cystic fibrosis.

C = Complications/additional conditions: H.T.=hypertension (secondary), E=emphysema, A=angina, B=bronchitis, Eo.=eosinophilia, My.=myelitis, C.P=cor pulmonale, As.=asthma, Ar.=arthritis, Db.=diabetes.

S = Smoker (/ = history of smoking, x = non-smoker)

R.T.= Radiotherapy (/ indicates patient recieved therapy before resection)

B = B-agonist therapy

St. = Long term steroid therapy.

H = / indicates patient was severely hypoxic for some time before the lung was removed.

R = Source of tissue, R = resection, PM = Post-mortem, T = transplant.

T = Time elapsed from removing the lung in theatre to fixing/freezing the lung in the laboratory.

APPENDIX 2.

Addresses of Suppliers (alphabetical listing).

Amersham International plc., U.K. Sales Office, Lincoln Place, Green End, Aylesbury, Bucks. HP20 2TP.

BDH (British Drug Houses) Chemicals Ltd., Freshwater Road, Dagenham, Essex RM8 1RZ.

Beckman R.I.I.C. Ltd., Sci.Instmt.Mfrs, Eastfield Industrial Estate, Glenrothes, Fife.

Brights Instrument Company, St. Margarets Way, Stokely Meadows, Huntingdon, Cambridgeshire. PE18 6EB.

British Oxygen Co. Ltd., 24, Deer Park Road, London SW19 3UF.

CRB Ltd. (Cambridge Research Biochemicals), Button End, Harston, Cambridge. CB2 5NX.

Dako Ltd., 22, The Arcade, High Wycombe, Bucks., HP11 2HT.

Difco Laboratories, P.O. Box 14B, Central Avenue, East Molesey, Surrey.

East Acres Biologicals, Box 822, 236, Blackmer Road, Southbridge, MA 01550, Massachussets, U.S.A.

Gibco Ltd., P.O. Box 35, 3, Washington Road, Paisley, Scotland. PA3 4EF.

Graticules Ltd., Sovereign Way, Botany Trading Estate, Tonbridge, Kent.

ICN Biomedicals Ltd., Free Press House, Castle Street, High Wycombe, Bucks., HP3 6RN.

Infrakem Ltd., Unit 51, Bradley Training Estate, Standish, Nr. Wigan, Lancs.

James Burrough FAD Ltd., 70, Eastways Industrial Park, Witham, Essex.

Janssen Life Science Products, Grove, Wantage, Oxon,. OX12 ODQ.

Lavenread Computors Ltd., 90, Portland Road, London. SE25 4PJ.

Leitz (Instruments) Ltd., 48, Park Street, Luton, LU1 3HP.

Pye-Unicam Ltd., York Street, Cambridge. CB1 2PX.

Raymond A. Lamb, 6 Sunbeam Road, London, NW10 6JL.

Reichert-Jung U.K. Ltd., 820, Yeovil Road, Slough, Berks. SL1 4JB.

Serendipity Systems Inc.: U.K. Distributors - Great Northern Computor Services Ltd., 116, Low Lane, Horsforth, Leeds, Yorkshire. LS18 5PX.

Sigma Chemical Company, Fancy Road, Poole, Dorset. BH17 7NH.

'Statgraphics' (Statistical Graphics Corporation), STSC Inc., 2115 East Jefferson Street, Rockville MD20852, Maryland, U.S.A.

Spencer's Paints and Wall Coverings PLC, Froghall, Aberdeen, Scotland.

Tandon Computor Co., 49, Statheam Place, Simi Valley, California 93065, U.S.A.

Ultraclone Ltd., P.O. Box 288, Cambridge. CB2 1NQ.

Wellcome Research Laboratories, Langley Court, Beckenham, Kent. BR3 3BS.

APPENDIX 3.

1. Staining schedules.

Haematoxylin and Eosin.

a. Cryostat sections 10-20u at -30°C.	
b. Fix in 10% neutral buffered formalin	10mins
c. Wash in running tap water.	3mins
d. Immerse in Harris's haematoxylin	45secs
e. Rinse in distilled water and 'blue' in running tap water	3mins
f. Immerse in 1% aqueous eosin	10secs
g. Rinse in 95% ethanol	3secs
h. Dehydrate in 70%, 90%, 2x100% ethanol	4x3mins
i. Clear in xylene	2x3mins
j. Mount in DPX.	

Alcian Blue/Periodic acid Schiff,

a. Air dry cryostat sections and post-fix in neutral buffered formalin	10mins
b. Dewax paraffin sections and bring to water	
c. Rinse in distilled water	2mins
d. Immerse in 3% acetic acid	2mins
e. Stain in 1% Alcian blue (pH 2.5)	5mins
f. Wash in running tap water	30mins
g. Treat with 0.3% sodium carbonate	30mins
h. Rinse in distilled	20mins
i. Immerse in 1% aqueous periodic acid	5mins
j. Rinse in running tap water	5mins
k. Immerse in Schiff's reagent	3mins
l. Rinse x2 in sulphurous acid rinse	2x2mins
m. Wash in running tap water to 'pink'	5-10mins
n. Dehydrate in graded ethanol, clear and mount.	

Acidic glycoproteins (predominantly found in mucus cells) are stained blue and neutral carbohydrate components (found in serous cells), pink.

2. Counterstaining schedules.

Haematoxylin.

a. Rinse sections in distilled water	2x1 min
b. Immerse in Harris's haematoxylin	20secs
c. Rinse briefly in distilled water	
d. Differentiate in acid-alcohol	10secs
e. Immerse in running tap water to 'blue'	3mins
f. Dehydrate, clear and mount.	

Alcian blue, haematoxylin and eosin.

a. Rinse sections in distilled water	2x1 min
b. Immerse in Alcian blue (pH 2.5)	2mins
c. Rinse in running tap water	10mins
d. Immerse in Harris's haematoxylin	20secs
e. Rinse briefly in distilled water	
f. 'Blue' in running tap water	3mins
g. Immerse in 1% aqueous eosin	10secs
h. Immerse briefly in 95% ethanol	
i. Dehydrate, clear and mount.	

Mucous containing cells are stained turquoise blue, nuclei, blue/purple and cytoplasm, pink.

All Stains and chemicals were purchased from BDH Ltd.

APPENDIX 4.

General information about commercial antisera used and other reagents used in immunohistochemical methods.

1. Primary antisera.

Antibody	Immunogen	Donor	Supplier
PGP 9.5	not known	Rabbit	Ultraclone
NSE	not Known	Rabbit	Dako
VIP	natural Porcine VIP	Rabbit	ICN
SP	Substance P (1-11)	Rabbit	CRB
CGRP	Synthetic rat CGRP (1-37)	Rabbit	CRB
NPY	Synthetic NPY (1-36)	Rabbit	CRB
Tyrosine Hydroxylase	not known	Rabbit	East Acres Biologicals.

2. Secondary antisera.

Antibody	Donor	Supplier.
FITC-conjugated anti-rabbit IgG	Pig	ICN
Biotinylated anti-rabbit IgG	Pig	Dako
FITC-avidin	/	Dako
Unconjugated anti-rabbit IgG	Goat	Dako
PAP complex (Rabbit IgG)	Rabbit	Dako
Gold-conjugated anti-rabbit IgG	Goat	Janssen

Lyphilised antisera were reconstituted in in distilled water and then diluted to 1:10 with buffer diluent (0.05M Tris buffered saline, pH 7.4 containing 0.1% bovine serum albumin and 0.01% sodium azide; the azide was omitted from antisera used in peroxidase-anti-peroxidase technique). Antisera were stored in aliquots of 500µl at -20°C and made up to working dilution as required.

3. Additional reagents.

All buffer reagents were purchased from BDH chemicals Ltd. Bovine serum albumin, free avidin and free biotin, and DAB reagent were purchased from Sigma chemical company. All non-immune animal sera were supplied by Dako Ltd.

REFERENCES

Adams G.K., Peters S.P. and Lichtenstein L.M. 1984. Arachiodonic acid metabolism and airway smooth muscle. In: Kay A.B., Austin K.F. and Lichtenstein L.M. (eds.) Asthma: Physiology, Immunology, Pharmacology and Treatment. London Academic Press. 101-2.

Aherne W.A. and Dunnil M.S. 1982. Morphometry. Edward Arnold Ltd. London

Al-Bazzaz F.J. and Kelsey J. 1982. Effect of substance P on ion transport by tracheal mucosa. Am. Rev. Resp. Dis. 125, 243.

Alton E.W., Batten J., Hodson M., Wallwork J., Higenbottam T. and Geddes D.M. 1987. Absence of electrochemical defect of cystic fibrosis in transplanted lung. The Lancet, i, 1026.

Alexander H.L. 1933. The autonomic control of heart, lungs and bronchi. Ann Intern. Med. 6, 1033.

Alquist R.P. 1948. A study of the adrenotropic receptors. J. Physiol. 153, 587-600.

Anderson D.H. 1938. Cystic fibrosis of the pancreas and its relation to celiac disease. A clinical and pathological study. Am. J. Dis. Child 56, 344-399.

Anderson D.H. 1962. Pathology of cystic fibrosis. Ann. NY Acad. Sci. 95, 500-517.

Andersson R.G.G. and Grundstrom N. 1983. The excitatory non-cholinergic, non-adrenergic nervous system of guinea-pig airways. Eur. J. Resp. Dis. 64, 141-47.

Andersson R.G.G., Grundstrom N. and Lindgren B.R. 1985b. An alpha-2-adrenoceptor-mediated inhibition of the excitatory noncholinergic neurotransmission in the airways and the neurogenic vasodilation and extravasation in the skin. In: Tachykinin antagonists pp 189-197. Hakanson R. and Sundler F. (eds.) Elsevier, (Amsterdam.)

Ariens E.J. and Simonis A.M. 1976. Receptors and receptor mechanisms. In: β-Adrenoceptor Blocking Agents. Saxena P.R and Forsythe R.P. (eds.), Amsterdam, Elsevire, pp3-27.

Assem E.S.K. and Schild H.O. 1969. β-adrenergic receptors concerned with the anaphylactic mechanisms. Int. Arch. Allergy 45, 62-69.

Augustinsson K.B. 1968. The evolution of esterases in vertebrates. In: Homologous Enzymes and Biochemical Evolution. N. Van Thoai and J. Roche (eds) Gordon and Breach, (New York.) 299-311.

Austin L. and Phillis J.W. 1965. The distribution of cerebellar cholinesterases in several species. J. Neurochem. 12, 709-717

Axelsson S., Bjorklund A. and Lindvall O. 1972. Fluorescence histochemistry of biogenic monoamines. A study of the capacity of various carbonyl compounds to form fluorophores with biogenic monoamines in gas phase reactions. J. Histochem. Cytochem. 20, 435-444.

Baker B., Peatfield A.C. and Richardson P.S. 1985. Nervous control of mucin secretion into human bronchus. J. Physiol. (Lond.), 365, 297-305.

Baker D.G., Basbaum C.B., Herbert D.A. and Mitchell R.A. 1983. Transmission in airway ganglia of ferrets: inhibition by norepinephrine. Neurosci. Lett. 41, 139-43.

Barnes P.J. 1985. Adrenoceptors in bronchial asthma. In Szabadi E., Bradshaw C.M. and Nahorski S.R. (eds.), Pharmacology of adrenoceptors. (London), MacMillan, 205-14.

Barnes P.J. 1986a. Neural control of human airways in health and disease. Am. Rev. Resp. Dis. (suppl.) 134, 1289-1314.

Barnes P.J. 1986b. Endogenous catecholamines and asthma. J. Aller. Clin, Immunol. 77, 791-5.

Barnes P.J. 1986c. Asthma as an axon reflex. Lancet 1, 242-5.

Barnes P.J. 1987. Airway neuropeptides and airway disease. Ann. Ital. Med. Int. II, No.4, 327-332.

Barnes P.J. 1984. The third nervous system in the lung: Physiololgy and clinical perspectives. Thorax 39, 561-7.

Barnes P.J., Basbaum C.B. and Nadel J.A. 1983. Autoradiographic localisation of autonomic receptors in airway smooth muscle: marked differences between large and small airways. Am. Rev. Resp. Dis. 127, 758-62.

Barnes P.J., Dewar A. and Rogers D.F. 1986. Human bronchial secretion: effect of substance P, muscarinic and adrenergic stimulation in vitro. Br. J. Pharmacol. 89, 767P

Barnes P.J. and Dixon C.M.S. 1984. The effect of inhaled vasoactive intestinal peptide on bronchial hyperreactivity in man. Am. Rev. Resp. Dis. 30, 162-6.

Barnes P.J., Ind P.W. and Brown M.J. 1982. Plasma histamine and catecholamines in stable asthmatic subjects. Clin. Sci. 62, 661-5.

Barnes P.J., Ind P.W. and Dollery C.T. 1984. β-adrenoceptors in asthma and their response to agonists. In: Kay A.B., Austin K.F. and Lichtenstein L.M. (eds) Asthma: Physiology, Immunology, Pharmacology and Treatment. London Academic Press 1984, 339-58.

Barnes P.J., Maclagan J. and Meldrum L.A. 1987. Effects of tachykinins on

cholinergic neural responses in guinea-pig trachea. Br. J. Pharmacol. 90, 138P

Bartholinus T. 1663. De pulmonum substantia et motu diatribe. Accedunt Marcelli Malpighii de pulmonibus observatione anatomicae. Hafniae (Copenhagen) H. Giodani.

Belcher G. and Ryall R.W. 1977. Substance P and Renshaw cells: a new concept of inhibitory synaptic interactions. J. Physiol. (Lond.) 272, 105-19.

Bench K.G., Gordon G.B. and Miller W.R. 1965. Studies on the bronchial counterpart of the Kultschitzky (Argentaffin) cell and innervation of bronchial glands. J. Ultrastruct. Res. 12, 668-686.

Bergner A.D. and Bayliss M.W. 1952. Histochemical detection of fatal anticholinesterase poisoning. U.S. Armed Forces Med. J. 3, 1637-1644.

Berkley H.J. 1893. The intrinsic pulmonary nerves by the silver method. J.Comp.Neurol. 3, 107

Berkin K.G., Inglis G.C., Ball S.G. and Thompson N.C. 1985. Airway responses to low concentrations of adrenaline and noradrenaline in normal subjects. Q. J. Exp. Physiol. 70, 203-209.

Berry J.L. 1935. The relation between bronchial amnd pulmonary circulation in the human lung, investigated by radiopaque injections. J. Physiol. (Lond.), 24, 305-14.

Berthelsen S. and Pettinger W.A. 1977, A functional basis for classification of alpha-adrenergic receptors. Life Sci. 21, 595-606.

Birdsall N.J., Hulme E.C., Stockton J., Burgen A.S., Bernie C.P., Hammer R., Wong E.H. and Zigmond M.J. 1983. Muscarinic receptor subclasses: evidence from binding studies. Adv. Biochem. Psychopharmacol. 37, 323.

Bishop A.E., Polak J.M., Bloom S.R. and Pearse A.G.E. 1978. A new universal technique for the immunocytochemical localisation of peptidergic innervation. J. Endocrinol. 77, 25P-26P.

Bishop A.E., Polak J.M., Lake B., Bryant M.G. and Bloom S.R. 1980a Abnormalities of neural and hormonal peptides in Hirchsprung's disease. Reg. Peptides (suppl.) 1, 11

Bishop A.E., Polak J.M., Bryant M.G., Bloom S.R. and Hamilton S. 1980b, Abnormalities of Vasoactive Intestinal Polypeptide-containing nerves in Crohn's disease. Gastroent. 79, 853-860.

Black J., Turner A. and Shaw J. 1981. Alpha-adrenoceptors in human peripheral lung. Eur.J.Pharmacol. 72, 83-86.

Bloom S.R. and Edwards A.V. 1980. Vasoactive intestinal peptide in relation

to atropine resistant vasodilatation in the submaxillary gland of the cat. J. Physiol. 300, 41-53.

Borson D.B., Charlin M., Gold B.D. and Nadel J.A. 1980. Neural regulation of $^{35}SO_4$-macromolecule secretion from traceal gland of ferrets. J. Appl. Physiol. 57, 457-66.

Boyd E.M. 1972. Respiratory Tract Fluid. Springfield IL: Thomas.

Bradley G.W. 1977. Control of the breathing pattern. Widdicome J.G. (ed) Respiratory Physiology II vol.14 Univ. Park Press. (Balt.) 185-217.

Brain S.D., Williams T.J., Tipins J.R., Morris H.R. and MacIntyre I. 1985. Calcitonin gene-related peptide is a potent vasodilator. Nature. 313, 54-56.

Brown M.J., Jenner D.A., Allison D.J. and Dollery C.T. 1981. Variations in individual organ release of noradrenaline measured by an improved radioenzymatic technique; limitations of peripheral venous measurements in the assessment of sympathetic nervous activity. Clin Sci. 61, 585-90.

Buch S.H. and Burcher E. 1986. the tachykinins: a family of peptides with a brood of 'receptors'. T.I.P.S., 65-68.

Buchanan K.D., Kerr J.I., Johnston C.F., Redmond A.O.B. and Craig B.G. 1983. The diffuse endocrine system in cystic fibrosis. Scand. J. Gastroenterol. 82, (suppl.) 155-63.

Burnstock G., Campbell G., Bennett M. and Holman M.E. 1963. Inhibition of the smooth muscle of Taenia coli. Nature 200, 581-82.

Burnstock G. 1969. Evolution of the autonomic innervation of visceral and cardiovascular systems in vertebrates. Pharmacol Rev. 21, 247-324.

Burnstock G. 1972. Purinergic nerves. Pharmacol Rev. 24, 509-81

Burnstock G. 1976. Do some nerve cells release more than one transmitter? Neurosci. 1, 239-48.

Burnstock G. 1977. Autonomic neuroeffector junctions - reflex vasodilation of the skin. J. Invest. Dermatol. 69, 47-57.

Burnstock G. 1978. A basis for distinguishing two types of purinergic receptor In: Cell membrane receptors for drugs and hormones : A multidisciplinary approach. pp. 107-118 Straub R.W. and Bolis L. (eds.) Raven Press (New York).

Burnstock G. and Griffith S.G. 1983. Innervation of microvascular smooth muscle. Prog. Appl. Microcirc. 3, 19-39.

Butchers P.R., Skidmore I.F., Vardey C.J. and Wheeldon A.M. 1980. Characterization of the receptor mediation, the anti-anaphylactic effects

of beta-adrenoceptor agonists in human lung tissue in vitro. Br. J. Pharmacol. 71, 663-7.

Cabezas G.A., Grof P.D. and Nadel J.A. 1971. Sympathetic versus parasympathetec nervous regulation of airways in dogs. J. Appl. Physiol. 31, 651.

Cadieux A., Springall D.R., Mulderry P.K., Rodrigo J., Ghatei M.A., Terenghi G., Bloom S.R. and Polak J.M. 1986. Occurance, distribution and ontogeny of CGRP immunoreactivity in the rat lower respiratory tract: effect of capsaicin and treatment and surgical denervations. Neurosci. 19, No.2, 605-627.

Cameron A.R., Johnston C.D., Kirkpatrick C.T. and Kirkpatrick M.C.A. 1983. The quest for the inhibitory neurotransmitter in bovine tracheal smooth muscle. Quart. J. Exp. Psychol. 68, 413-26.

Carstairs J. 1987. Distribution of CGRP-receptors in the lung. Eur. J. Pharmacol. 140, 357-8.

Carstairs J. and Barnes P.J. 1986a. Autoradiographic mapping of substance P receptors in lung. Eur. J. Pharmacol. 127, 295-6.

Carstairs J.R. and Barnes P.J. 1986b. Visualisation of vasoactive intestinal peptide receptors in human and guinea-pig lung. J. Pharmacol. Exp. Ther. 239, 249.

Carstairs J.R., Nimmo A.J. and Barnes P.J. 1985. Autoradiographic visualisation of beta-adrenoceptor subtypes in human lung. Am Rev. Resp. Dis. 132, 541-47.

Chatonnet A. and Masson P. 1984. The peptidase site of butyryl-cholinesterase is distinct from the esteratic site. C.R. Acad. Sc. Paris, t299, Serie III, No.13, 529-34.

Christensen N.J. 1982. Sympathetic nervous activity and age. Eur. J. Clin. Invest. 12, 91-92.

Chubb I.W., Hodgson A.J. and White. 1980. Acetylcholinesterase hydrolyses substance P. Neurosci. 5, 2065-2072.

Clarke P.B.S., Schwartz R.D., Paul S.M., Pert C.B. and Pert A. 1985. Nicotinic binding in rat brain: autoradiographical comparison of [^{3}H]acetylcholine, [^{3}H]nicotine and [^{125}I]-alpha-bungarotoxin. J.Neurosci. 5, 1307-1315.

Coburn R.F. 1984. The anatomy of the ferret paratracheal parasympathetic nerve-ganglion plexus. Exp. Lung Res. 7, 1-9.

Coburn R.F. and Tomita T. 1973. Evidence for non-adrenergic inhibitory

nerves in the guinea-pig trachealis muscle. Am. J. Physiol. 224, 1072-80

Coleman R.A. 1976. Effects of some purine derivatives on the guinea-pig trachea and their interaction with drugs that block adenosine uptake. Br. J. Pharmac. 57, 51-57.

Coleman R.A. 1980. Purine antagonists in the identification of adenosine-receptors in guinea-pig trachea and the role of purines in non-adrenergic inhibitory neurotransmission. Br.J. Pharmacol. 69, 359-366.

Coleman R.A. and Levy G.P. 1974. Non-adrenergic inhibitory nervous pathway in guinea-pig trachea. Br. J. Pharmacol. 52, 167-74.

Coleridge J.C.G. and Coleridge H.M. 1984. Afferent vagal C-fibre innervation of the lungs and its functional significance. Rev. Physiol. Biochem. Pharmacol. 99, 1-110.

Coles S.J., Neill K.H. and Reid L.M. 1984. Potent stimulation of glycoprotein secretion in canine trachea by substance P. J. Appl. Physiol. 57, 1323-7.

Coles S.J., Said S.I. and Reid L.M. 1981. Inhibition by VIP of glycoconjugate and lysozyme secretion by human airways in vitro. Am.Rev.Resp.Dis. 124, 531-6.

Comsa J., Leonhardt H. and Werkerle H. 1982. Hormonal coordination of the immune response. Rev. Physiol. Biochem. Pharmacol. 92, 169-91.

Conneally P.M., Merritt A.D. and Yu P. 1973. Cystic fibrosis: population genetics. Tex. Rep. Biol. Med. 31, 639-650.

Cook R.D., and Burnstock G. 1976. The ultrastructure of Auerbach's plexus in the guinea-pig trachea. Br. J. Pharmacol. 52, 171-194.

Coons A.H., Creech H.J. and Jones R.N. 1941. Immunological properties of an antibody containing a fluorescent group. Proc. Soc. Exp. Biol. Med. 47, 200-202.

Coons A.H. and Kaplan M.H. 1950. Localisation of antigen in tissue cells. J. Exp. Med. 91, 1-13.

Coons A.H., Leduc E.H. and Connolly J.M. 1955. Studies on antibody production 1. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. J. Exp. Med. 102, 49-59.

Cotrell G.A. and Macon J.B. 1974. Synaptic connexions of two symmetrically placed giant serotonin-containing neurons. J.Physiol. 236, 435-464.

Cuello A.C. 1982. Co-transmission. Macmillan Press, (London).

Cutz E., Chan W. and Track N.S. 1981. Bombesin, calcitonin and leu-

enkephalin like immunoreactivity in endocrine cells of human lung. Experentia 37, 765-7

Dale H.H. 1914. The action of certain esters and ethers of choline and their relation to muscarine. J. Pharm. Exp. Ther. 6, 190-97.

Daly J.W. 1982. Adenosine receptors: targets for future drugs. J. Med Chem. 25, 197.

Daly M de B. and Hebb C. 1966. Pulmonary and Bronchial Vascular Systems. Edward Arnold Ltd. (London).

Daniel E.E., Kannan C., Davis C., and Posey Daniel V. 1986. Ultrastructural studies on the neuromuscular control of human tracheal and bronchial muscle. Resp. Physiol. 63, 109-128.

Danks D.M., Allan J. and Anderson C.M. 1965. A genetic study of fibrocystic disease of the pancreas. Ann. Hum Genetic. 28, 323-356.

Danscher G. 1981. Localisation of gold in biological tissue. A photochemical method for light and electron microscopy. Histochemistry 71, 81.

Das S., Edwardson J.A., Hughes D. and McDermott. 1982. Butyryl-cholinesterase (BuChE)- positive glial cells in the pituitary intermediate lobe: evidence for their role in the formation of pituitary colloid and that BuchE is an endopeptidase. J.Physiol. London. 327, 45.

Das R.M., Jeffery P.K. and Widdicome J.G. 1979. The eplithelial innervation of the lower respiratory tract of the cat. J.Anat. 126, 123.

Davis B., Marin M., Fischer S., Graf P., Widdicome J. and Nadel J.A. 1976. New method for study of canine mucous gland secretion in vivo: cholinergic regulation. Am.Rev. Resp. Dis. 113, 257.

Davis P.B., Boat T.F., Stern R.C. and Doershuk C.F. 1982b. Beta-adrenergic responses are normal in cystic fibrosis. Clin. Res. 30, 749A.

Davis P.B., Braunstein M. and Jay C. 1978. Decreased adenosine 3':5'-monophosphate response to isoproterenol in cystic fibrosis leukocytes. Pediatr. Res. 12, 703-707.

Davis P.B. and Kaliner M. 1983. Autonomic nervous system abnormalities in cysic fibrosis. J. Chron. Dis. 36, No.3, 269-278.

Davis C. and Kannan M.S. 1987. Sympathetic innervation of human tracheal and bronchial smooth muscle. Resp. Physiol. 68, 53-61.

Davis C., Kannan S., Jones T.R. and Daniel E.E. 1982a. Control of human airway smooth muscle: in vitro studies. J.Appl.Physiol: Resp. Environ. Exer. Physiol. 53, No.5, 1080-87.

Davis P.B., Shelhamer J. and Kaliner M. 1980. Abnormal adrenergic and

cholinergic sensitivity in cystic fibrosis. N. Engl. J. Med. 302, 1453-1456.

Dearborn D.G. 1976. Water and electrolytes of exocrine secretions. In: Mangos A.J. and Talamo R.C. (Eds), Cystic Fibrosis: Projections into the Future. Strattan Int. Med. Book Corp. (New York). 179-191.

De La Torre J.C. and Surgeon J.W. 1976. A methodological approach to rapid and sensitive monoamine histofluorescence using a modified glyoxylic acid technique: the SPG method. Histochemistry. 41, 335-353.

De Monchy J.G.R., Kauffman H.F., Venge Per et al. 1985. Bronchoalveolar eosinophilia during allergen-induced late asthmatic reaction. Am. Rev. Resp. Dis. 131, 373-6.

Denniss A.R. and Young J.A. 1978. Modification of salivary duct electrolyte transport in rat and rabbit by pysalaemin, VIP, GIP and other enterohormones. Pfleugers Arch. 376, 73.

Dey R.D., Shannon Jr. W.A. and Said S. 1981. Localization of VIP-immunoreactive nerves in airways and pulmonary vessels of dogs, cats and human subjects. Cell. Tiss. Res. 220, 231-238.

Doidge J.M. and Satchell D.G. 1982. Adrenergic and nonadrenergic inhibitory nerves in mammalian airways. J. Auton. N. Syst. 5, 83-99.

Doran J.F., Jackson P., Kynoch P.A. and Thompson R.J. 1983. Isolation of PGP 9.5. a new human neurone-specific protein detected by high resolution two-dimensional electrophoresis. J. Neurochem. 40, 1542-7.

Dressel T.D., Goodale R.L., Borner B.A. and Etani S. 1980. A study of the cholinesterases of the canine pancreatic sphincters and the relationship between reduced butyrylcholinesterase activity and pancreatic ductal hypertension. Ann. Surg. 192, 614-619.

Easson L.H. and Stedman E. 1937. The specificity of choline-esterase. Biochem. J. 31, 1723-1729.

Eaton J.A. and Fedde M.R. 1977. Identification of two populations of biogenic amine-containing cells in the mouse lung. Cell. Tiss. Res. 176, 243-249.

Ecobichon D.J. and Comeau A.M. 1973. Pseudocholinesterases of mammalian plasma: physicochemical properties and organophosphate inhibition in eleven species. Toxicol. Appl. Pharmacol. 24, 92-100.

Edvinsson L., Emson P., McCulloch J., Tatemoto K. and Uddman R. 1983 Neuropeptide Y: cerebrovascular innervation and vasomotor effects in the cat. Neurosci. Lett. 43, 79-84.

Ehrlich P. 1885. Zbl. Med. Wiss. 23, 113

Ekblad E., Edvinsson L., Wahlstedt C., Uddman R., Hakanson R. and Sundler F. 1984. Neuropeptide Y co-exists and co-operates with noradrenaline in peripheral vascular nerve fibres. Regl. Peptides 8, 225-35.

Eklund B., Jogestrand T. and Pernow B. 1977. Effects of substance P on resistance and capacitance vessels in the human forearm. In: von Euler U.S. and Pernow B. (eds) Raven Press, (New York). 275-85.

El-Badawi A. and Schenk E.A. 1967. Histochemical methods for separate, consecutive and simultaneous demonstration of acetylcholinesterase and norepinephrine in cryostat sections. J. Histochem. Cytochem. 15, 580-588.

El-Bermani A.L. 1973. Innervation of the rat lung. Acetylcholinesterase-containing nerves of the bronchial tree. Am.J. Anat. 137, 19.

El-Bermani Al-WI. 1978. Pulmonary noradrenergic innervation of rat and monkey: a comparative study. Thorax. 33, 167.

Elde R., Holkfelt T., Johansson O. and Terenius L. 1976. Immunohistochemical studies using antibodies to leucine-enkephalin: initial observations on the nervous system of the rat. Neurosci. 1, 349-51.

Elftman A.G. 1943. The afferent and parasympathetic innervation of the lungs and trachea of the dog. Am. J. Anat. 72, 1.

Elliot T.R. 1904. On the action of noradrenaline. J. Physiol. 31, 20

Ellman G.L., Courtney D.K., Andre V . and Featherstone R.M. 1961. A new and rapid determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88-95.

Eppinger H. and Hess L. 1909. Zur Pathologie des vegitativen Nervensystems. Ztscher. f. Klin. Med. 67, 345.

Erjavec F., Lembeck F., Florjane-Irman T., Skofitsch G., Donnerer J., Saria A. and Holzer P. 1981. Release of histamine by substance P. Naunyn Schmied. Arch. Pharmacol. 317, 67-70.

Falck B. and Owman 1965. A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic amines. Acta Unibersitatis Lundensis, II, 7, 4-23.

Faulk W.R. and Taylor G.M. 1971. An immunocolloid method for the electron microscope. Immunocytochemistry 8, 1081-3.

Feldberg. W. 1945. Present veiws on the mode of action of acetylcholine in the central nervous system. Physiol Rev. 25, 596-642

Fillenz M. and Woods M.J. 1970. Sensory innervation of airways. In:

Breathing: Hering-Breuer Centenery Symposium. (Ciba Foundation Symp. Ed. R. Porter.) 101-9.

Finney M.J.B., Karlsson J.A. and Persson. 1985. Effects of broncho-constrictors and bronchodilators on a novel human small airway preparation. Br. J. Pharmacol. 85, 29-36.

Fischer A.W.F. 1964. The intrinsic innervation of the trachea. J.Anat. 98, 117.

Feyrter F. 1938. Uber Diffuse Endokrine Epitheliale Organe. J.A. Barth Leipzig p6.

Fox B., Bull T.B. and Guz A. 1980. Innervation of alveolar walls in the human lung: an electron microscopic study. J.Anat. 131, 683.

Franco-Cereceda A. and Lundberg J.M. 1987. Potent effects of neuropeptide Y and calcitonin gene-related peptide on human coronary vascular tone in vitro. Acta Physiol. Scand. 131, 159-60.

Frewtell C.M.S., Foreman J.C., Jordan C.C., Oheme P.P., Remer H. and Stewart J.M. 1982. The effects of substance P on histamine and 5-hydroxy-tryptamine release in rat. J. Physiol. (London) 330, 393-44.

Frizzell R.A., Rechkemmer G. and Shoemaker R.L. 1986. Altered regulation of airway epithelial cell chloride channels in cystic fibrosis. Science 233, 558-60.

Fukuda T. and Koelle G.B. 1959. The cytological localization of intracellular neuronal acetylcholinesterase. J. Biophys. Biochem. Cytol. 5, 433-440.

Fuller R.W., Dixon C.M.S. and Barnes P.J. 1985. The bronchoconstrictor response to inhaled capsaicin in humans. J. Appl. Physiol. 85, 1080.

Fuller R.W., Dixon C.M.S., Dollery C.T. and Barnes P.J. 1986. Inflammatory mediator interaction in asthma: prostaglandin D_2 potentiation of histamine and cholinergic hypperesponsiveness. Am. Rev. Resp. Dis. 133, 252-4.

Fuller R.W., Dixon C.M.S., Cuss F.M.C. and Barnes P.J. 1987. Bradykinin-induced bronchoconstriction in man: mode of action. Am.Rev. Resp.Dis. 135, 176.

Fuller R.W., Maxwell D.L., Dixon C.M.S. et al 1987. The effects of substance P on cardiovascular and respiratory function in human subjects. J. Appl. Physiol. 62, 1473-79.

Furness J.B. and Costa M. 1975. The use of glyoxylic acid for the fluorescence histochemical demonstration of peripheral stores of noradrenaline and 5-hydroxytryptamine in whole mounts. Histochemistry 41, 335-352.

Galant S.P., Norton L., Hebst J. and Wood C. 1981. Impaired beta-adrenergic receptor binding and function in cystic fibrosis neutrophils. J. Clin. Invest. 68, 253-258.

Gallager J.T., Kent P.W., Passatore M., Phipps R.J. and Richardson P.S. 1975. The composition of tracheal mucous and the nervous control of its secretion in the cat. Proc. R. Soc. London Ser. B192, 49-76.

Gashi A.A., Borson D.B., Finkbeiner W.E., Nadel J.A. and Basbaum C.B. 1986. Neuropeptides degranulate serous cells of ferret tracheal glands. Am. J. Physiol. 251, C223-9.

Gaylor J.B. 1934. The intrinsic nervous mechanism of the human lung. Brain. 57, 143-160.

Geoghegan W.D., Scillian J.J. and Ackerman G.A. 1978. The detection of human B-lymphocytes by both light and electron microscopy utilising colloidal gold-labelled anti-immunoglobulin. Immunol. Commun. 7, 1-12.

Ghatei M.A., Sheppard M.N., O'Shaughnessy D.J., Adrian T.E., McGregor G.P., Polak J.M. and Bloom S.R. 1982. Regulatory peptides in the mammalian respiratory tract. Endocrinol. 111, 1248-54.

Gibbins L.L. 1982. Lack of correlation between ultrastructural and pharmacological types of non-adrenergic autonomic nerves Cell Tiss Res. 221, 551-581.

Gidelli B., Buffa R., Salvini P., DeRai P., Uggeri F., Fiocca R. and Said S.I. 1982. Lack of VIP-immunoreactive nerves in oesophageal aschalasia. Ital. J. Gastroenterol. 14, 211-213.

Gomori G. 1948. Histochemical demonstration of sites of choline esterase activity. Proc. Soc. Exptl. Biol. Med. 68, 354-358.

Graham R.C. and Karnovsky M.J. 1966. The early stages of absorption of injected horse raddish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. 14, 291-302.

Grandordy B.M., Rhoden K.J., Fossard N. and Barnes P.J. 1987. Tachykinin receptors and phosphoinositide turnover in airways. Am. Rev. Resp. Dis. 135, A87.

Gray T.S. and Morley J.E. 1986. Neuropeptide Y: anatomical distribution and possible function in mammalian nervous system. Life Sci. 38, 389-401.

Greenberg B., Rhoden K. and Barnes P.J. 1987. Vasoactive intestinal peptide causes non-endothelial dependent relaxation in human and bovine pulmonary arteries. Blood Vessels 24, 45-50.

Grieco M.H. and Pierson R.N. 1971. Mechanism of bronchoconstriction due to

B-adrenergic blockade. J. Allergy Clin. Immunol. 48, 143-52

Grieg M.E. and Holland W.C. 1949a. Studies on the permeability of erythrocytes. I. The relationship between cholinesterase activity and permeability of dog erythrocytes. Arch. Biochem. Biophys. 23, 370-84.

Grieg M.E. and Holland W.C. 1949b. Increased permeability of the hemoencephalic barrier produced by physostigmine acetylcholine. Science 110, 237.

Grillo M.A. 1966. Electron microscopy of sympathetic tissues. Pharmacol. Rev. 18, 387-399.

Grube D. 1980. Immunoreactivities of gastrin (G) cells. II. Non-specific binding of immunoglobulins to G-cells by ionic interactions. Histochemistry 66, 149-167.

Grundstrom N., Andersson R.G.G. and Wikberg J.E.S. 1981. Pharmacological characterization of the autonomous innervation of the guinea-pig tracheobronchial smooth muscle. Acta Pharmacol. Toxicol. 49, 150-157.

Grundstrom N. and Andersson R.G.G. 1985. In vivo demonstration of alpha-2-adrenoceptor-mediated inhibition of the excitatory non-cholinergic neurotransmission in the guinea-pig airways. Naunyn-Schmiedeberg's Arch. Pharmacol. 328, 236-240.

Gu J., De Mey J., Moreman M. and Polak J.M. 1981. Sequential use of the PAP and immunogold staining methods for the light microscope double staining of tissue antigens. Reg. Peptides 1, 365-374.

Gu J., Islam K.N. and Polak J.M. 1983. Repeated application of first layer antiserum improves immunofluorescence staining: a modification of the indirect immunofluorescence procedure. Histochemical J. 15, 475-482.

Hage E. 1980. Light and electron microscopic character of the various lung endocrine cell types. Invest. Cell. Pathol. 3, 345.

Hage E., Hage J. and Juel G. 1977. Endocrine-like cells of the pulmonary epithelium of the human adult lung. Cell Tiss. Res. 178, 39-48.

Hahn H.L., Wilson A.G., Graf P.D., Fischer S.P. and Nadel J.A. 1978. Interaction between serotonin and efferent vagus nerves in dog lungs. J. Appl. Physiol. 44, 144-9.

Hall B.D. and Simpkiss M.J. 1968, Incidence of fibrocystic disease in Wessex. J. Med. Genet. 5, 262-265.

Hammer R. and Giachetti A. 1982 Muscarinic receptor subtypes M1 and M2: Biochemical and functional characterisation. Life Sci. 31, 2991-2998

Hartman B.K. 1973. Immunofluorescence of dopamine β-hydroxylase.

Application of improved methodology to the localisation of the peripheral and central noradrenergic nervous system. J. Histochem. Cytochem. 21, 312-332.

Hawes R.C. and Alles G.A. 1940. Cholinesterase in the blood of allergic patients. J. Allergy 12, 1.

Hebb C.O. 1957. Biochemical evidence for the neural function of acetylcholine. Physiol. Rev. 37, 196-220.

Hedlung B., Abens J. and Barfai T. 1983. Vasoactive intestinal polypeptide and muscarinic receptors: supersensitivity induced by long-term atropine treatment. Science 220, 519.

Heggeness M.H. and Ash J.F. 1977. Use of the avidin-biotin complex for the localisation of actin and myosin with fluorescence microscopy. J. Cell Biol. 73, 783-88

Heinz-Erian P., Dey R.D., Flux M. and Said S.I. 1985. Deficient Vasoactive Intestinal Peptide innervation in the sweat glands of cystic fibrosis patients. Science 229, 1407-08.

Henderson W.R., Shelhamer J.R., Reingold D.B., Smith L.J., Evans R. III. and Kaliner M. 1979. α-adrenergic hyperresponsiveness in asthma: analysis of vascular and pupillary responses. New Engl. Med. J. 300, 6427.

Hirsch E.F., Kaiser A.C., Borner H.B., Cooper T. and Rains J.J. 1968. Innervation of the mammalian lung I. The afferent receptors, Arch. Path. 85, 51.

Hogg J.C., Mackelm P.T. and Thurlbeck W.M. 1968. Site and nature of airway obstruction in chronic obstructive lung disease. New Engl. J. Med. 278, 1355-60.

Holgate C.S., Jackson P., Cowen P.N. and Bird C.C. 1983. Immunogold-silver staining : new method of immunotaining with enhanced sensitivity. J. Histochem. Cytochem. 31, 938-944.

Holkfelt T., Johansson O., Ljungdahl A., Lundberg J.M. and Schultzberg M. 980. Peptidergic neurones. Nature 284, 515-521.

Holzer P. and Lippe I.T. 1984. Substance P can contract the longitudunal muscle of the guinea-pig small intestine by releasing intracellular calcium. Br. J.Pharmacol. 82, 259-67.

Holtzman M.J., Sheller J.R., Dimeo M. Nadel J.A. and Boushey H.A. 1980. Effect of ganglionic blockade on bronchial reactivity in atopic subjects. Am. Rev. Resp. Dis. 122, 17-25.

Hornykiewicz O. 1973. Dopamine in the basal ganglia: its role and theraputic implications (including the clinical use of L-DOPA). Br. Med. Bull. 29,

172-178.

Hoyes A.D., Barber P. and Jagessar H. 1982. Location in the nodose ganglion of the perikarya of neurons whose axons distribute in the epithelium of the rat rachea. J.Anat (London) 134, 265-71.

Ind P.W., Brown M.J. and Barnes P.J. 1983. Sympathoadrenal reponses in asthma. Thorax 38, 702.

Ind P.W., Causon R.C., Brown M.J. and Barnes P.J. 1985. Circulating catecholamines in acute asthma. Br. Med. J. 290, 267-79.

Ito Y. and Tokeda K. 1982. Nonadrenergic inhibitory nerves and putative transmitters in the smooth muscle of cat trachea. J. Physiol. 330, 497-511.

Iversen L.L. 1967. The Uptake and Storage of Noradrenaline in Sympathetic Nerves. Cambridge University Press, (London).

Jacobsen C.F., Leonis J., Linderstrom-Lang K. and Ottesen M. 1957. The pH-stat and its use in biochemistry. In: Methods of biochemical analysis, Vol IV, D. Glick (ed) Interscience Publishers (New York). pp171-210.

Jacobwitz D., Kent K.M., Fleisch J.H. and Cooper T. 1973. Histofluorescent study of catecholamine-containing elements in cholinergic ganglia from the calf and dog lung. Proc. Soc. Exp. Biol. Med. 144, 464.

Jansco N., Jansco-Gabor A. and Szolcsanyi L. 1968. The role of sensory nerve endings in neurogenic inflammation induced in human skin and in the eye and paw of the rat. Br. J. Pharmacol. Chemother. 32, 32-41.

Jeffery P.K. 1982. The innervation of bronchial mucosa. In: Cellular Biology of the Lung. Cumming G. and Bonsignore G. (eds.) Plenum Press, London, 1-32.

Jeffery P.K., Nelson F.C., Wardlaw A., Kay A.B. and Collins J.V. 1987. Ultrastructural analysis of bronchial mucosa in asthma. J. Pathol. 151, 58A.

Jeffery P.K. and Reid L. 1973. Intra-epithelial nerves in normal rat airways: a quantitative electron microscopic study. J. Anat. 131, 413.

Kaliner M., Orange R.P. and Austin K.F. 1972. Immunological release of histamine and SRS-A from human lung IV. Enhancement by cholinergic and α-adrenergic stimulation. J.Exp. Med. 136, 556-67.

Kaliner M., Shelhamer J., Davis P.B., Smith L.J. and Ventner J.C. 1982. Autonomic nervous system abnormality and allergy. Ann. Int. Med. 96,

349-57.

Karlsson J.A. and Pearsson C.G.A. 1984. Neither Vasoactive Intestinal Peptide nor purine derivatives may mediate nonadrenergic tracheal inhibition. Acta Physiol Scand. 122, 589-98.

Karlsson J.A., Finney M.J.B., Persson C.G.A. and Post C. 1984. Substance P antagonist and the role of tachykinins in non-cholinergic bronchoconstriction. Life Sci. 35, 2681-91.

Karnovsky M.J. and Roots L. 1964. A 'direct coloring' thiocholine method for cholinesterases J. Histochem. Cytochem. 12, 219-221.

Katz B. and Thesleff S. 1957. A study of the 'desensitization' produced by acetylcholine at the motor end-plate. J. Physiol. 138, 63-80.

Kawatani M., Rutigliano M. and De Groat W.C. 1985. Depolarisation and muscarinic excitation induced in a sympathetic ganglion by vasoactive intestinal polypeptide. Science 229, 879.

Kiernan J.A. 1975. A pharmacological and histological investigation of the involvement of mast cells in cutaneous axon reflex vasodilation. Q. J. Exp. Physiol. 60, 123-30.

King A.S., McLelland J., Cook R.D., King D.A. and Walsh C. 1974. The ultrastructure of afferent nerve endings in the avian lung. Respir. Physiol. 22, 21-40.

Koelle G.B. 1950. The histochemical differentiation of types of cholinesterases and their localizations in tissues of the cat. J. Pharmacol. Exptl. Therap. 100, 158-179.

Koelle G.B., Koelle E.S. and Friedenwald J.S. 1950. The effect of specific and non-specific cholinesterase on the motility of the isolated ileum. J. Pharmacol. Exp. Ther. 100, 180-91.

Koelle G.B. 1951. The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distributions. J. Pharmacol. Exptl. Therap. 103, 153-171.

Koelle G.B. and Friedenwald J.S. 1949. A histochemical method for localizing cholinesterase activity. Proc. Soc. Exptl. Biol. Med. 70, 617-622.

Kneussel M.P. and Richardson J.B. 1978. Alpha-adrenergic receptors in human and canine tracheal and bronchial smooth muscle. J. Appl. Physiol. 45, 307-11.

Knight D.S. 1980. A light and electron microscopic study of feline intrapulmonary ganglia. J. Anat. 131, 413.

Knowlton G.C. and Larrabee M.G. 1946. A unitary analysis of pulmonary

volume receptors. Am.J. Physiol. 147, 100-114.

Kreijs G.J., Fordtran J.S., Fahrenkrug J., Schaffalitzky de Muckadell O.B., Fischer J.E., Humphrey C.S., O'Dorisio T.M., Said S.I., Walsh J.H. and Shulkes A.A. 1980. Effect of VIP infusion on water and ion transport in the human jejunum. Gastroenterol. 78, 722-7

Laitenen A. 1985a. Autonomic innervation of the human respiratory tract as revealed by histochemical and ultrastructural methods. Eur. J. Resp. Dis. 66, No.140 (suppl) 1-42.

Laitenen A. 1985b. Ultrastructural organisation of intraepithelial nerves in the human airway tract. Thorax 40, 488-92.

Laitenen L.A., Heino M., Laitenen A., Kava T. and Haahtela T. 1985. Damage of the airway epithelium and bronchial reactivity in patients in patients with asthma. Am. Rev. Resp. Dis. 131, 599-606.

Laitenen L.A., Laitenen A. Panula P.A., Partanen M., Tervo K. and Tervo T. 1983. Immunohistochemical demonstration of substance P in the lower respiratory tract of the rabbit and not of man. Thorax 38, 531-6.

Laitenen L.A., Laitenen A., Salonen R.O. and Widdicombe J.G. 1987a. Vascular actions of airway neuropeptides. Am. Rev.Resp. Dis. 136, (suppl.) 59-64.

Laitenen L.A., Laitenen A. and Widdicombe J.G. 1987b. Parasympathetic nervous control of tracheal vascular resistance in the dog. J.Physiol (London) 385, 135-46.

Laitenen A., Partanen M., Hervonen A. and Laitenen L. 1985. Electron microscopic study on the innervation of the human lower repiratory tract: Evidence of adrenergic nerves. Eur. J. Resp. Dis. 67, 209-15.

Lake C.R., Davis P.B., Zieler M. and Kopin L.J. 1979. Electrolytes and norepinephrine levels in the blood of patients with cystic fibrosis. Clin. Chim. Acta 92, 537-39

Lamb D. and Reid L. 1972a. The tracheobronchial submucosal glands in cystic fibrosis: a qualitative and quantitative histochemical study. Br.J. Dis Chest 66, 239.

Langer S.Z. 1974. Presynaptic regulation of catecholamine release. Biochem. Pharmacol. 23, 1793-800.

Langley J.N. 1898. On inhibitory fibres in the vagus for the end of the oesophagus and the stomach. J. Physiol. 23, 407-414.

Langley J.N. and Anderson H.K. 1895, The innervation of the pelvic and adjoining viscera. IV. The internal generative organs. J. Physiol. 19, 122-

130.

Larsson K., Martinsson A. and Hjemdahl P. 1982. Sympathoadrenal reactivity in exercise induced asthma. Chest 82, 561-7.

Larsell O. 1922. The ganglia, plexuses and nerve-terminations of the mammalian lung and pleura pulmonalis. J. Comp. Neurol. 35, 97.

Larsell G. and Dow R.S. 1933. The innervation of the human lung. Am. J. Anat. 52, 125-146.

Lauweryns J.M. and Cokelaere M. 1973. Hypoxia-sensitive neuro-epithelial bodies. Intrapulmonary secretory neuroreceptors, modulated by the CNS. Zeitschrift fur Zellforschung und mikroskopische Anatomie 145, 521-540.

Lauweryns J.M., De Bock V., Gulinckx P. and Decramer M. 1983. Effects of unilateral hypoxia on neuroepithelial bodies in rabbit lungs. J. Appl. Physiol: Resp. Environ. Exer. Physiol. 55, 1665-68.

Lauweryns J.M. and Godeeris P. 1975. Neuroepithelial bodies in the human child and adult lung. Am. Rev. Resp. Dis. 111, 469-476.

Lauweryns J.M. and Peuskens J.C. 1972. Neuroepithelial bodies (neuroreceptor or secretory organs?) in human infant bronchial and bronchiolar epithelium. Anat. Rec. 172, 471-481.

Lauweryns J.M. and Van Lommel A. 1986. Effect of various vagotomy procedures on the reaction to hypoxia of rabbit neuroepithelial bodies: modulation by intrapulmonary axon reflexes? Exp. Lung Res. 11, 319-39.

Lauwereyns J.M., Van Lommel A.T. and Dom R.J. 1985. Innervation of rabbit intrapulmonary neuroepithelial bodies: quantitative and qualitative ultrastructural study after vagotomy. J. Neurol. Sci. 67, 81-92.

Lauweryns J.M. and Van Ranst L. 1987. Calcitonin gene-related peptide immunoreactivity in rat lung: light and electron microscopic study. Thorax 42, 183-189.

Lefkowitz R.J. 1978. Regulation of β-adrenergic receptors by β-adrenergic agonists. In: Receptors and Hormone action. Birnbaumer L. and O'Malley B.W. (eds) vol.3 Academic Press, (New York). 179-194.

Legay C., Saffrey M.J. and Burnstock G. 1982. Co-existance of immunoreactive substance P and serotonin in nerones of the gut. Brain Res. 302, 379-382.

Lemanske R.F., Mischeler E.H., Farrell P.M., Anderson C. and Bulse W.W. 1981. Analysis of granulocyte beta-adrenergic response in cystic fibrosis: correlation of decreased responsiveness with disease severity. Am Rev. Resp. Dis. 123, 622-626.

Lembeck F. and Holzer P. 1979. Substance P as neurogenic mediator of

antidromic vasodilation and neurogenic plasma extravasation. Naunyn-Schmiedeberg. Arch. Pharmacol. 310, 175-183.

Lewis P.R. 1961. The effect of varying the conditions in the Koelle technique. Biblioteca Anat. 2, 11-20.

Lieberman J. and Rodbard S. 1975. Low blood pressure in young adults with cystic fibrosis. An effect of chronic salt loss in sweat? Ann Int Med. 82, 806-808.

Loewi. O. 1921. Uber humorale Ubertragbarkeit der Herznervenwirkung. Pflugers Arch. 189, 239-242

Lummis S.C.R. and Satelle D.B. 1985. Binding of N-[propionyl-^{3}H]propionylated alpha bungarotoxin and L- [benzilic- 4, 4'- ^{3}H] quinuclidyl benzylate to CNS extracts of the cockroach Periplaneta americana. Comp. Biochem. Physiol. [C]: 80(1) 75-83

Lundberg J.M., Anggard A., Fahrenkrug J., Holkfelt T. and Mutt V. 1980. Vasoactive intestinal polypeptide in cholinergic neurons of exocrine glands: Functional significance of coexisting transmitters for vasodilation and secretion. Proc. Natl. Acad. Sci. USA. 77, 1651.

Lundberg J.M., Anggard A., Emson P., Fahrenkrug J. and Holkfelt T. 1981. Vasoactive intestinal polypeptide and cholinergic mechanisms in cat nasal mucosa: studies on choline acetyltransferase and release of vasoactive intestinal polypeptide. Proc. Natl. Acad. Sci. USA. 78, 5255-9.

Lundberg J.M., Brodin E. and Saria A. 1983a. Effects and distribution of vagal capsaicin sensitive substance P neurons with special reference to the trachea and lungs. Acta Physiol. Scand. 119, 243-52

Lundberg J.M., Franco-Cereda A., Hua X., Holkfelt T. and Fiscer J.A. 1985a. Co-existence of substance P and calcitonin gene-related peptide-like immunoreactivities in sensory nerves in relation to cardiovascular and broncho-constrictor effects of capsaicin. Eur. J. Pharmacol. 108, 315-319.

Lundberg J.M., Holkfelt T., Martling C.R., Saria A and Cuello C. 1984. Substance P immunoreactive sensory nerves in the lower respiratory tract of various mammals including man. Cell Tiss. Res. 235, 251-61.

Lundberg J.M., Martling C.R. and Saria A. 1983b. Substance P and capsaicin induced contraction of human bronchi. Acta Phys. Scand. 119, 49-53.

Lundberg J.M. and Saria A. 1982. Capsaicin-sensitive vagal neurons involved in control of vascular permeability in rat trachea. Acta Physiol. Scand. 115, 521-23.

Lundberg J.M. and Saria A. 1983. Capsaicin induced desensitization of the

airway mucosa to cigarette smoke, mechanical and chemical irritants. Nature 302, 251-3.

Lundberg J.M., Saria A., Brodin E., Rosell S. and Folkers K. 1985b. Differential effects of reserpine and 6-hydroxydopamine on neuropeptide Y (NPY) and noradrenaline in peripheral neurons. Naunyn-Schmiedeberg's Arch. Pharmacol. 328, 331-340.

Lundberg J.M., Saria A., Brodin E., Rusells S. and Folkes R. 1983c. A substance P antagonist inhibits vagally induced increases in vascular permeability and bronchial smooth muscle contraction in guinea-pig. Proc. Natl. Acad. Sci. U.S.A. 80, 1120-4.

Lundberg J.M., Saria A., Theodorsson-Norheim E., Brodin E., Hua X.Y. 1985c. Multiple tachykinins in capsaicin-sensitive afferents: occurance, release and biological effects with special refernce to irritation of the airways. In: Tachykinin Antagonists. Hakanson F., Sundler F., (eds.), Amsterdam, Elsevier. pp159-69.

Lundberg J.M. and Stjarne L. 1984. Neuropeptide Y (NPY) depresses the secretion of ^{3}H-noradrenaline and the contractile response evoked by field stimulation in rat vas deferens. Acta Physiol. Scand. 120, 477-479.

Lundberg J.M., Terenius L., Hokfelt T., Martling C.R., Tatemoto K., Mutt V., Polak J., Bloom S. and Goldstein M. 1982. Neuropeptide-Y (NPY)-like immunoreactivity in peripheral noradrenergic nerons and effects of NPY on sympathetic function. Acta Physiol. Scand. 116, 477-480.

Lundblad L. 1984. Protective reflexes and vascular effects in the nasal mucosa elicited by activation of capsaicin-sensitive substance P immunoreactive trigeminal neurons. Acta Physiol. Scand. (suppl) 529, 1-42.

Lundblad L., Anggard A., and Lundberg J.M. 1984. Effects of antidromic trigeminal nerve stimulation in relation to parasympathetic vasodilation in cat nasal mucosa. Acta Physiol. Scand. 119, 7-13.

Lundblad L., Lundberg J.M., Brodin E. and Anggard A. 1983. Origin and distribution of sensory substance P nerves in the nasal mucosa. Acta Otolayngol. 96, 485-93.

Lung M.A., Wang J.C.C. and Cheng K.K. 1976. Bronchial circulation: an autoperfusion method for assessing its vasomotor activity and the study of -adrenoreceptors in the bronchial artery. Life Sci. 19, 577-80.

Malm L., Sundler F. and Uddman R. 1980. Effects of vasoactive intestinal polypeptide on resistance and capacitance vessels in the nasal mucosa.

Acta Otolaryngol. 90, 304-8.

Malmgrem H. and Sylven B. 1955. On the chemistry of the thiocholine method of Koelle. J. Histochem. Cytochem. 3, 441-445.

Mann S.P. 1971. The innervation of mammalian bronchial smooth muscle: the localization of catecholamines and cholinesterases. Histochem. J. 3, 319.

Mann J.S. and George C.F. 1985. Anti-cholinergic drugs in the treatment of airways disease. Br. J. Dis. Chest 79, 209-28.

Marangos P.J., Schmechel D.E. 1980. The neurobiology of the brain enolases. In: Essays in Neurochemistry and Neuropharmacology. Vol 4. Youdim M.B.H., Lovenberg W., Sharman D.F. and Lagnado J.R., (eds) (New York), John Wiley and Sons, 211-247.

Martinson J. 1965. Studies on the efferent vagal control of the stomach. Acta Physiol. Scand. (suppl.) 255, 1-23.

Martinson J. and Muren A. 1963. Excitatory and inhibitory effects of vagus stimulation on gastric motility in the cat. Acta Physiol. Scand. 57, 309-316.

Martling C.R., Gazelius B. and Lundberg J.M. 1987. Nervous control of blood flow to the cat trachea. Acta Physiol. Scand. 130, 409-17.

Martling C.R., Theodorsson-Norheim E. and Lundberg J.M. 1987. Occurance and effects of multiple tachykinins (substance P, neurokinin A and neuropeptide K) on human bronchi. Life Sci. 40, 1633-43.

McGaff C.J. and Milnor W.R. 1982 Effects of serotonin on pulmonary blood volume in the dog. Am.J. Physiol. 202, 457-460.

McKuisick V.A. 1978. Mendelian inheritence in man: catalogs of autosomal dominant, autosomal recessive and X-linked phenotypes. 5th ed. (Baltimore) John Hopkins Univ. Press.

McPherson M.A., Dormer R.L. and Dodge J.A. and Goodchild M.C. 1985. Adrenergic secretory responses of submandibular tissues from control subjects and cystic fibrosis patients. Clin. Chim. Acta 148, 229-237.

Mendel B., Mundell D.B. and Rudney H. 1943. Studies on cholinesterse 3. Specific tests for true cholinesterase and pseudo-cholinesterase. Biochem. J. 37, 473-476.

Mendel B. and Myers D.K. 1955. Identification of pseudocholinesterase in the tissues of ruminants. Nature. 176, 783-784.

Messer J.W., Peters A. and Bennet W.A. 1960. Causes of death and pathologic findings in 304 cases of bronchial asthma. Dis. Chest. 38, 616-24.

Meyrick B. and Reid L. 1970. Ultrastructure of cells in the human bronchial

submucosal glands. J.Anat. 107, 281.

Michel H.O. 1949. An electrometric method for the determination of red blood cell and plasma cholinesterase activity. J. Lab. Clin Med. 34, 119-125.

Mills J.E., Sellick H. and Widdicome J.G. 1970. Epithelial irritant receptors in the lungs In: R.Porter (ed) Breathing: Hering-Breuer Centenary Symposium. Churchill, (London) 77-92.

Mitchell L, Corey M., Woenne R., Krastins L.R.B. and Levison H. 1978. Bronchial hyperreactivity in cystic fibrosis and asthma. J. Pediatr. 93, 744-748.

Morice A. and Sever P. 1986. Vasoactive intestinal peptide as a bronchodilator in severe asthma. Peptides 7, 279-280.

Morice A., Unwin R.J. and Sever P.S. 1984. Vasoactive intestinal peptide as a bronchodilator in asthmatic subjects. Peptides 5, 439-40.

Myers D.K. 1953, Studies on Cholinesterase 9. Species variation in the specificity pattern of the pseudo cholinesterases. Biochem. J. 55, 67-79.

Nadel J.A. 1980. Autonomic regulation of airway smooth muscle. In: Physiology and Pharmacology of airways. Vol. 15 Lung Biology in Health and Disease J.A. Nadel (ed) P. Debber. pp217-257.

Nadel J.A. and Barnes P.J. 1984. Autonomic regulation of the airways. Annu. Rev. Med. 35, 451-67.

Nadel J.A., Salem H., Tamplin B. and Tokiwa Y. 1965. Mechanism of bronchoconstriction during inhalation of sulfur dioxide. J. Appl. Physiol. 20, 164-7.

Nadel J.A., Widdicombe J.H. and Peatfield A.C. 1985. Regulation of airway secretions, ion movements and water movements. American Physiological Society Handbook of Physiology: The Respiratory System I. Washington D.C.: American Physiological Society. 419-45.

Nahorski S.R. 1978. Heterogenity of cerebral beta-adreoceptor binding sites in various vertebrate species. Eur. J. Pharmacol. 51, 199-209.

Nagaishi C. 1972. Functional Anatomy and Histology of the Lung. University Park Press, (Balt).

Nakane P.K. and Pierce C.B. Jr. 1966. Enzyme-labelled antibodies: preparation and application for the localisation of antigens. J. Histochem. Cytochem. 14, 929-31.

Nathanson I., Widdicome J.H. and Barnes P.J. 1983. Effect of vasoactive intestinal peptide on ion transport across the dog tracheal epithelium.

J.Appl. Physiol. 55, 1257-61.

Nilsson G., Dahlberg K., Brodin E., Sundler F. and Strandberg K. 1977. Distribution and constrictor effect of substance P in guinea-pig tracheobronchial tissue. In: Substance P. Von Euler U.S. and Pernow B. (eds.) (New York) Raven Press. 75-81.

O'Donnell S.R., Saar N. and Wood L.J. 1978. The density of adrenergic nerves at various levels in the guinea-pig lung. Clin. Exp. Pharmacol. Physiol. 5, 325-32.

Oppenheimer E.H. 1981. Similarity of the tracheobronchial mucous glands and epithelium in patients with and without cystic fibrosis. Human Pathol. 12, 36-

Oppenheimer E.H. and Esterly J.R. 1973. Cystic fibrosis of the pancreas: morphological findings in infants with and without diagnostic pancreatic lesions. Arch. Pathol. 96, 149-154.

Osborne N.N. 1983. Dale's Principle and Communication Between Neurones. Pergamon Press, (Oxford)

Pack R.J. and Richardson P.S. 1984. The aminergic innervation of the human bronchus: a light and electron microscopic study. J.Anat. 138, 493-502.

Pack R.J. and Widdicome J.G. 1984. Amine-containing cells of the lung. Eur.J. Dis. 65, 559-578.

Palmer J.B., Cuss F.M.C. and Barnes P.J. 1986a. VIP and PHM and their role in nonadrenergic inhibitory responses in isolated human airways. J. Appl. Physiol. 61, 1322-28

Palmer J.B., Cuss F.M.C. and Barnes P.J. 1986b. Sensory neuropeptides and human airway function. Am. Rev. Resp. Dis. 133, A113.

Palmer J.B., Cuss F.M.C., Mulderry P.K., Ghatei M.A., Springall D.R., Cadieux A., Bloom S.R., Polak J.M. and Barnes P.J. 1987. Calcitonin gene-related peptide is localised to human airway smooth muscle. Br. J. Pharmacol. 91, 95-101.

Palmer J.B.D., Cuss F.M.C., Warren J.B. and Barnes P.J. 1986c. The effect of infused vasoactive intestinal peptide on airway function in normal subjects. Thorax 41, 663-6.

Partanen M., Hervonen A., Toivanen M., Laitinen A. and Laitinen L.A. 1982. The innervation of human lower respiratory tract as revealed by catecholamine histofluorescence and neuropeptide immunohistochemistry. International Conference on Bronchial Hyperreactivity. Conference

Summary. (Oxford). The Medicine Publishing Foundation. pp73-74.

Pauwels R. and Van Der Straeten M. 1986. The bronchial effects of adenosine in the rat. Arch. Int. Pharmacodyn. 280, (Suppl), 229-239.

Payan G.P., Levin J.D. and Goetzl E.J. 1984. Modulation of immunity and hypersensitivity by sensory neuropeptides. J. Immunol. 132, 1601-4.

Pearse A.G.E. 1969. The cytochemistry and ultrastructure of polypeptide-hormone producing cells of the APUD series and the embryologic, physiologic and pathologic implications of the concept. J. Histochem. Cytochem. 17, 303.

Peatfield A.C. 1980. The control of mucus secretion into the lumen of the trachea. London: London Univ., Dissertation.

Peatfield A.C., Barnes P.J., Bratcher C., Nadel J.A. and Davis B. 1983. VIP stimulates tracheal submucosal gland secretion in ferret. Am. rev. Resp. Dis. 128, 89-93.

Peatfield A.C. and Richardson P.S. 1983. Evidence for non-cholinergic, non-adrenergic nervous control of mucus secretion into the cat trachea. J. Physiol. (London) 342, 335-45.

Peatfield A.C. and Richardson P.S. 1982. The control of mucin secretion into the lumen of the cat trachea by α- and β-adrenoceptors, and their relative inolvement during sympathetic nerve stimulation. Eur. J. Pharmacol. 81, 617-26.

Pernow J., Ohlen A., Holkfelt T., Nilsson O. and Lundberg J.M. 1987. Neuropeptide Y: presence in perivascular noradrenergic neurons and vasoconstrictor effects on skeletal muscle blood vessels in experimental animals and man. Regul. Peptides 19, 313-324.

Persson C.G.A., Erjefalt L. and Karlsson J.A. 1985. Effects of tachykinins and tachykinin antagonists on the tracheobronchial microcirculation. In: Tachykinin Antagonists. Hakanson R., Sundler F. (eds.), Amsterdam, Elsevier. pp171-79.

Pessaq T.P. 1971. The innervation of the lung of newborn children. Acta Anat. 79, 93-101

Phipps R.J., Nadel J.A. and Davis B. 1980. Effect of alpha-adrenergic stimulation on mucus secretion and on ion-transport in the cat trachea in vitro. Am. Rev. Resp. Dis. 121, 359-65.

Phipps R.J., Richardson P.S., Corfield A., Gallagher J.T., Jeffery P.K., Kent P.W. and Passatore M. 1977. A physiological, biochemical and histological study of goose tracheal mucin and its secretion. Philos. Trans. R. Soc. London. 279, 513-40.

Polak J.M. and Bloom S.R. 1982. Regulatory peptides in the respiratory tract of man and other animals. Exp. Lung Res. 3, 313-328.

Polak J.M. and Bloom S.R. 1986. Regulatory peptides of the gastrointestinal and respiratory tracts. Int. Arch. Pharmacol. 280, (Suppl.), 16-49.

Polak J.M. and Van Noorden S. 1983. Immunocytochemistry: Practical Applications in Pathology and Biology. J.Wright and Sons Ltd. (Bristol).

Potter D.D., Furshpan E.J. and Landis S.C. 1983. Transmitter status in cultured rat sympathetic neurons: plasticity and multiple function. Fed. Proc. 42, 1626-1632.

Quigley C.J., Fuller R.W., Dixon C.M.S. and Barnes P.J. 1985. Inhaled acetylcholinesterase inhibitors cause dose related bronchoconstriction in asthmatic and normal subjects. Am. Rev. Resp. Dis. 131, A283.

Quinton P.M. 1982. Abnormalities in electrolyte secretion in cystic fibrosis sweat glands due to decreased anion permeability. In: Fluid and Electrolyte Abnormalities in Exocrine Glands in Cystic Fibrosis. Quinton P.M., Martinez J.R. and Hopfer V. (eds.) San Francisco Press Inc. (CA). 94101-6800.

Quinton P.M. and Bijman J. 1983. Higher bioelectric potentials due to decreased chloride absorption in the sweat glands of patients with cystic fibrosis. New Engl. J. Med. 308, 1185-1189.

Raaijmakers J.A.M., Harmsen H.J., De Boer E., Beneker C. and van Rozen R.J. 1986. Phospholipase A_2 products induce pulmonary β-adrenoceptor deviations. Eur. J. Pharmacol. 127, 163-64.

Raaijmakers J.A.M., Terpstra G.K., van Rozen R.J., Witter A. and Kreukniet J. 1984. Muscarinic cholinergic receptors in peripheral lung tissue of normal subjects and of patients with chronic obstructive lung disease. Clin. Sci. 66, 585-90.

Raeburn D., Hay D.W.P., Farmer S.G. and Fedan J.S. 1986. Epithelium removal increases the reactivity of human isolated tracheal musacle to methacholine and reduces the effect of verapamil. Eur. J. Pharmacol. 123, 451-3.

Reed C.E. 1974. Abnormal autonomic mechanisms in asthma. J. Allergy Clin. Immunol. 53, 34-41.

Reid L. 1960. Measurement of the bronchial mucus gland layer: a diagnostic yardstick in chronic bronchitis. Thorax 15, 132-141.

Reid L. and DeHaller R. 1967. The bronchial mucous glands- their

hypertropy and change in intracellular mucous. Mod. Probl. Pediatr. 10, 195-199.

Rhodin J.A. 1973. Nervous tissue-nerve endings. In: Histology. (London) Oxford Univ. Press. 294-312.

Rhodin J.A. 1966. Ultrastucture and function of the human tracheal mucosa. Am. Rev. Resp. Dis. 93, 1.

Richardson K.C. 1966. Electron microscopic identification of autonomic nerve endings. Nature, 210, 756.

Richardson J.B. 1979. Nerve supply to the lungs. Am. Rev. Resp. Dis. 119, 785-802

Richardson J.B. 1981. Nonadrenergic inhibitory innervation of the lung. Lung 159, 315-22

Richardson J.B. and Beland J. 1976. The nonadrenergic inhibitory nervous system in human airways. J. Appl. Physiol. 41, 764-771.

Richardson J.B. and Ferguson C.C. 1979. Morphology of the airways. In: Physiology and Pharmacology of the Airways J. Nadel (ed), Vol.15 of Lung Biology in Health and Disease. P. Debber.

Richardson P.S and Sterling G.M. 1969. Effects of beta-adrenergic receptor blockade on conductance and lung volume in normal and asthmatic subjects. Br. Med. J. 3, 143-5.

Robberecht P., Chatelain P., DeNeef P., Camus J.C., Waelbroek M. and Christophe J. 1981. Presence of vasoactive intestinal peptide receptors coupled to adenylate cyclase in rat lung membranes. Biochim. Biophys. Acta. 678, 76-82.

Roberts J.A., Raeburn D., Roger L.W. and Thomson N.C. 1984. Comparison of in vivo airway responsiveness and in vitro smooth muscle sensitivity to methacholine. Thorax 39, 837-43.

Rocholz E. 1957. Kinderlied und Kinderspiel aus der Schweiz. Weber Leipzig p280.

Rosell S. 1978. Nervous control of the microcirculation. In: Microcirculation. Kaley, Altura (eds). Univ. Park Press. (Baltimore). vol 2 371-400.

Rubin L.S., Barbero G.J. and Chernick W.S. 1966. Pupillary dysfunction as a concomitant of cystic fibrosis. Pediatr. 38, 865-73.

Sagor G.R., Baron J.H., Bloom S.R. and Polak J.M. 1982. The clinical relevance of regulatory peptides. Recent Adv. in Surgery. 1-25.

Said S.I. and Mutt V. 1970. A polypeptide with broad biological activity

isolated from small intestine. Science N.Y. 169, 1217-18.

Sant'Ambrogio G. 1982. Information arising from the tracheobronchial tree of mammals. Physiol Rev. 62, 531-569.

Saria A., Martling C.S., Dalsgaard C.J. and Lundberg J.M. 1985. Evidence for substance P spinal afferents that mediate bronchoconstriction. Acta Physiol Scand. 125, 407-414.

Satchell D. 1982. Non-adrenergic, non-cholinergic nerves in mammalian airways: their function and the role of purines. Comp. Biochem. Physiol. 72C, 189-196.

Sato K. 1977. The Physiology, Pharmacology and Biochemistry of the Eccrine Sweat Gland. Rev. Physiol. Biochem. Pharmacol. 79, 51-131

Sato K. and Sato F. 1984. Defective beta-adrenergic response of cystic fibrosis sweat glands in vivo and in vitro. J. Clin Invest. 73, 1763-1771.

Scheller J.R., Holzman M.J., Skoogh B.E. and Nadel J.A. 1982. Interaction of serotonin with vagal and acetylcholine induced bronchoconstriction in canine lungs. J. Appl. Physiol. 52, 964-6

Schmechel D.E., Marangos P.J., Brightman M.W. 1979. Neuron specific enolase is a marker for peripheral and central neuroendocrine cells. Nature 276, 834-836.

Schwachman H. and Mohmoodian A. 1979. Quality of sweat test performance in diagnosis of CF. Clin. Chem. 25, 158-161.

Scudamore H.H., Vorhaus L.J. and Kark R.M. 1954. Acetylcholine and cholinesterase in the blood of patients suffering with bronchial asthma. J. Lab. Med. 37, 860.

Shelhamer J.H., Marom Z., and Kaliner M. Immunologic and neuropharmacologic stimulation of mucuos glycoprotein release from human airways in vitro. J. Clin. Invest. 66, 1400-1408.

Shelhamer J.H., Marom Z., Sun F., Bach M.K. and Kaliner M. 1982. The effects of arachinoids and leukotrines on the release of mucus from human airways. Chest 81, (suppl) 36-37.

Sharma R.K. and Jeffery P.K. 1986. Indications of β-adrenoreceptor deficiency in lungs of patients with cystic fibrosis. Clin. Sci. 72, (suppl.16) 52P.

Sheppard M.N., Kurian S.S., Henzen-Logmans S.C., Michetti F., Cocchia D., Cole P., Rush R.A., Marangos P.J., Bloom S.R. and Polak J.M. 1983. Neuron-specific enolase and S-100: new markers for delineating the innervation of the respiratory tract in man and other mammals. Thorax 38, 333-40.

Sheppard M.N., Marangos P.J., Bloom S.R., and Polak J.M. 1984a. Neuron-specific enolase: A marker for the early development of nerves and endocrine cells in the human lung. Life Sci. 3, 265-71.

Sheppard M.N., Polak J.M., Allen J.M. and Bloom S.R. 1984b. Neuropeptide tyrosine (NPY): A newly discovered peptide is present in the mammalian respiratory tract. Thorax 39, 326-30.

Sibinga M.S. and Barbero G.J. 1961. Studies in physiology of sweating in cystic fibrosis 1. Experimental sweat gland fatigue. Pediatr. 27, 912-920.

Silver A. 1974. The biology of cholinesterases. In: Frontiers of Biology vol.36. Neuberger A. and Tatum E.L. (eds.) North Holland Research Monographs, North Holland Publishing Co. (Amsterdam).

Simonsson B.G., Jacobs F.M. and Nadel J.A. 1967. Role of the autonomic nervous system and cough reflex in increased responsiveness of airways in patients with chronic obstructive airway disease. J. Clin. Invest. 46, 1812-18.

Skidmore I.F. and Vardey C.J. 1984. The mediators of bronchial asthma and the mechanism of their release. Agents and Actions (Suppl.) 14, 33-58.

Skoogh B-E. 1983. Transmission through airway ganglia. Eur. J. Respir. Dis. 64, (suppl.), 159-70.

Skoogh B-E. 1986. Parasympathetic ganglia in the airways. Bull.Eur. Physiopathol. Respir. 22, (suppl.7), 143-7.

Skofitch G., Savitt J.M. and Jacobowitz D.M. 1985. Suggestive evidence for a functional unit between mast cells and substance P fibres in the rat diaphragm and mesentary. Histochemistry 82, 5-8.

Spencer H. and Leof D. 1964. The innervation of the human lung. J. Anat. (London), 123, 55.

Spock A., Heick H.M.C., Cress H. 1967. Abnormal serum factor in patients with cystic fibrosis of the pancreas. Pediatr. Res. 1, 173-177.

Springall D.R., Hacker G.W., Grimelius L. and Polak J.M. 1984. The potential of the immunogold-silver staining method for paraffin sections. Histochemistry 81, 603-608.

Springall D.R., Polak J.M., Ghatei M.A., Lackie P. and Bloom S.R. 1984. Calcitonin gene-related peptide (CGRP), a new regulatory peptide widely distributed in lung. J. Path. 143, 306-7.

Stanisz A.J., Scicchitano R., Stead R., Matsuda H., Tomioka M., Denburg J. and Bienenstock J. 1987a. Neuropeptides and Immunity. Am. Rev. Resp. Dis. 136, (suppl) 48-51.

Stanisz A., Scicchitano R., Dazin P., Bienenstock J. and Payan D. 1987b.

Distribution of substance P receptors on murine spleen and Peyer's patch T and B cells. J. Immunol 139, 749-54.

Stelzer J., Bigby B.G., Stulbarg. 1986. Ozone-induced changes in bronchial reactivity to methacholine and airway inflammation in humans. J. Appl. Physiol. 60, 1321-6.

Sternberger L.A. 1979. Immunocytochemistry 2nd Ed. John Wiley and Sons. (New York).

Stretton C.D. and Barnes P.J. 1988. Modulation of cholinergic neurotransmission in guinea-pig trachea by neuropeptide Y. Br. J. Pharmacol. 93, 672-678.

Strickland K.P. and Thompson R.H.S. 1954. The effect of cholinesterase inhibitors in the leakage of potassium from brain slices. Biochem J. 58, xx.

Strickland K.P. and Thompson R.H.S. 1955. On the mechanism of potassium loss from brain slices induced by cholinesterase inhibitors. Biochem. J. 60, 468-75.

Sturgess J. and Reid L. 1972a. An organ culture study of the effect of drugs on the secretory activity of the human bronchial submucosal gland. Clin Sci. 43, 533.

Sturgess J. and Reid L. 1972b. Secretory activity of the human bronchial mucous glands in vitro. Exp Mol. Pathol. 16, 362.

Sundler F., Hakanson R., Ekblad E., Uddman R. and Wahlstedt C. 1986. Neuropeptide Y in the peripheral adrenergic and enteric nervous system. Int. Rev. Cytol. 102, 243-69.

Szentivanyi A. 1968. The β-adrenergic theory of atopic abnormality in bronchial asthma. J. Allergy 42, 203-32.

Tam E., Sheppard D., Epstein J., Ancie P. and Boushey H.A. 1983. Effect of increasing doses of ipatropium bromide on dose-response curve to isocapnic hyperventilation with cold air in asthmatic subjects. Am. Rev. Resp. Dis. 127, (suppl) 4, 257.

Tanaka D.T. and Grunstein M.M. 1984. Mechanisms of substance P-induced contraction of rabbit airway smooth muscle. J. Appl. Physiol. 57, 1551-57.

Taton G., Dalhaye M., Camus J.-C., de Neef P., Chatelain P., Robberecht P. and Christophe J. 1981. Characterization of the VIP- and secretin-stimulated adenylate cyclase system from human lung. Plugers Arch. 391, 178-182.

Tattersfield A.E., Leaver D.G. and Pride N.B. 1973. Effect of beta adrenergic blockade and stimulation on normal human airways. J. Appl. Physiol. 35, 613-19

Taylor S.M., Pare P.D. and Scellenberg R.R. 1984. Cholinergic and non-adrenergic mechanisms in human and guinea-pig airways. J. Appl. Physiol. 56, 958-65.

Taylor W. 1982. Feyter cells. In: Cellular Biology of the Lung. Cumming G. and Bonsignore G. (eds) (New York), Plenum Press.

Taylor W. 1977. Pulmonary argyrophil cells at high altitude. J. Path. 122, 137-44.

Taxi J. 1952. Action du formol sur l'activite de diverses preparations de cholinesterases. J. Physiol. (Paris) 44, 595-599.

Thompson R.J.,Doran J.F., Jackson P., Dhillon A.P. and Rode J. 1983. PGP 9.5 - a new marker for vertebrate neurons and neuroendocrine cells. Brain Res. 278, 224-228.

Thompson E.H. and Whittaker V.P. 1952. Cholinesterase activity and sodium transport in the human red cell. Biochem. Biophys. Acta. 9, 700-701.

Uddman R., Malm L. and Sundler F. 1980. The origin of vasoactive intestinal polypeptide (VIP) nerves in the feline nasal mucosa. Acta Otolaryngol. 89, 152-6.

Uddman R. and Sundler F. 1987. I. Sites and synthesis: neuropeptides in the airways: a review. Am. Rev. Resp. Dis. (suppl) 136, 53-58.

Uddman R., Alumets J., Densert O., Hakanson R. and Sundler F. 1978. Occurance and distribution of VIP nerves in the nasal mucosa and tracheobronchial wall. Acta. Otolaryngol. 86, 443.

Uddman R., Sundler E. and Emson P. 1984. Occurance and distribution of neuropeptide Y immunoreactive nerves in the respiratory tract and middle ear. Cell Tiss. Res. 237, 321-27.

Ukei L, German V.F. and Nadel J.A. 1980. Micropipette measurement of airway submucosal gland secretion: autonomic effects. Am. Rev. Resp.Dis. 121, 351-357.

Undem B.J., Dick E.C. and Buckner C.K. 1983. Inhibition by vasoactive intestinal peptide of antigen-induced histamine release from guinea-pig minced lung. Eur. J. Pharmacol. 88, 247-250.

Varga E. and Szoor A. 1971. The cholinesterase activity of myosin. In: Contractile Proteins and Muscle. (New York), M. Dekker. 263-71

Vermiere P.A. and Vanhoutte P.M. 1979. Inhibitory effects of catecholamines in isolated canine bronchial smooth muscle. J. Appl. Physiol. 46, 787-91.

Vizi E.S. 1979. Presynaptic modulation of neurochemical transmission. Prog. Neurobiol. 12, 681-86.

Von Euler U.S. 1946. Specific sympathomimetic ergone in adrenergic nerve fibres (sympathin) and its relation to adrenaline and nor-adrenaline. Acta. Physiol. Scand. 12, 73-79.

Wahlstedt C., Yanaihara N. and Hakanson R. 1986. Evidence for pre- and post-junctional receptors for neuropeptide Y and related peptides. Reg. Peptides 13, 307-18.

Walton J.N. 1982, Some common disorders of the autonomic nervous system. In: Essentials of Neurology. J.N. Walton Pittman Books Ltd. (London) 230-235.

Ward M.J., Fentem P.H., Roderick Smith W.H. and Davies D. 1981. Ipatropium bromide in acute asthma. Br. Med. J. (282), 590-600.

Warren J.B. and Dalton N. 1983. A comparison of the bronchodilator and vasopressor effects of exercise levels of adrenaline in man. Clin. Sci. 64, 475-9.

Watson S.P. and Downes C.P. 1983. Substance P induced hydrolysis of inositol phospholipids in guinea-pig ileum and rat hypothalamus. Eur. J. Pharmacol. 93, 245-53.

Webber S.E. and Widdicome J.G. 1987. The effect of vasoactive intestinal peptide on smooth muscle tone and mucus secretion from the ferret trachea. Br. J. Pharmacol. 91, 139-48.

Welsh M.J. and Leidtke C.M. 1986. Chloride and potassium channels in cystic fibrosis airway epithelia. Epithelial transport 1, Fed. Proc. 45, 149-154.

Wenner W.F. and Buhrmester C.C. 1937. Potassium and acetylcholine in the blood of rabbits in anaphylactic shock. J. Allergy 9, 85.

Westfall D.P., Stitzel R.E. and Rowe J.N. 1978. The post-junctional effects and neural release of purine compounds in guinea-pig vas deferens. Eur. J. Pharmacol. 50, 27-38.

Wharton J., Polak J.M., Bloom S.R., Ghatei M.A., Solcia E., Brown M.R. and Pearse A.G.E. 1978. Bombesin-like immunoreactivity in the lung. Nature 273, 769-770.

Widdicome J.G. 1981. Nervous receptors in the respiratory tract and lungs. In: Regulation of Breathing Part II. T.F. Hornbein (ed) Marcel Dekker (New York). 429-472.

Widdicome J.G. 1977. Respiratory reflexes and defence In: Respiratory Defence Mechanisms. Brain J.D., Proctor D.F., and Reid L. (eds) (New York) Marcel Dekker. 593-630.

Widdicome J.G. 1986. Sensory innervation of the lungs and airways. Prog. Brain Res. 67, 49-64.

Wiebel E.R. 1963. Principles of methods for the morphometric study of the lung and other organs. Lab. Invest. 12, 131.

Wiesner-Menzel L., Schulz B., Vakilzadeh F. and Czarnetzki B.M. 1981. Electron microscopical evidence for a direct contact between nerve fibres and mast cells. Acta Derm. Venereol. (Stockh.) 61, 465-9.

Wilson G.B.A. 1983. Ciliary dyskinesia factors produced by leucocytes. In: Lymphokines 8. (New York) Academic Press. 323-371.

Wood J.D. 1984. Enteric neurophysiology. Am. J. Physiol. 247, G:585-98.

Wood G.S. and Warnke R. 1981. Suppresion of endogenous avidin-biotin activity in tissues and its relevance to biotin-avidin detection systems. J. Histochem. Cytochem. 29, 1196-1204.

Yalow R.S. and Berson S.A. 1959. Assay of plasma insulin in human subjects by immunological methods. Nature 184, 1648-9.

Yankaskas J.R., Knowles M.R., Gatzy J.T. et al. 1985. Persistence of abnormal chloride ion permeability in cystic fibrosis nasal epithelial cells in heterologous culture. Lancet i: 954-956.

Yeates D.B., Sturgess J.M. and Kahn S.R. ET AL. 1976. Mucociliary transport in trachea of patients with cystic fibrosis. Arch. Dis. Child 51, 28-33.

Zaagsma J., Van Der Heijden P.J., Van Der Schaar M.W.G. and Bank C.M.C. 1984. Differentiation of functional adrenoceptors in human and guinea pig airways. Eur. J. Resp. Dis. 65, (suppl.135), 16-33.

Zaagsma J., Van Der Heijden P.J., Van Der Schaar M.W.G. and Bank C.M.C. 1983. Comparison of functional β-adrenoceptor heterogenity in central and peripheral airway smooth muscle of guinea-pig and man. J. Recep. Res. 3, 89-106.

Zueizer W.W. and Newton W.A. 1949. The pathogenesis of fibrocystic disease of the pancreas. A study of 36 cases with special reference to pulmonary lesions. Pediatr. 4, 53

Zygmunt L. 1983 In: Neurotransmitters and Drugs. Kruk, Christopher & Pycock (eds) 2nd edition, Croom Helm Ltd.