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**Nicotine as an Odorant**

**A Biochemical and Electrophysiological Study  
of Receptors for Nicotine in the Olfactory  
Epithelium of the Rat.**

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Submitted for the degree of PhD  
to the University of Warwick, Coventry.

This work was carried out in the laboratories of the  
Olfaction Research Group,  
Department of Chemistry, Warwick University.

September 1987

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## **Declaration**

I hereby declare that the work contained within this thesis is my own, except where stated in the text (see Chapter 2b in particular).

## **Bibliography**

This thesis was prepared following the "Guidelines to Assist Candidates in Matters Related to Their Work for a Higher Degree by Research and in the Preparation of Their Thesis", as published by the Department of Chemistry, Warwick University, dated October 1986.

The general format of the thesis and the accepted abbreviations used were as described for material submitted to the Biochemical Journal (see "Policy of the Journal and Instructions to Authors", Biochem. J. (1987) 241, 1-24).

The majority of the material within this thesis has been published or has been submitted for publication prior to the submission of this thesis as follows;

Chapter 1.

EDWARDS, D.A., MATHER, R.A., SHIRLEY, S.G. & DODD, G.H. (1987)

Evidence for an Olfactory Receptor which Responds to Nicotine

- Nicotine as an Odorant.

Experientia 43, 868-873

Chapter 2a.

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Spatial Variation in Response to Odorants on the Rat

Olfactory Epithelium.

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Chapter 2b.

SHIRLEY, S.G, POLAK, E.H., EDWARDS, D.A., WOOD, M.A. & DODD, G.H.(1987)

The Effect of Concanavalin A on the Rat Electro-olfactogram at Varying  
Odorant Concentrations.

Biochem. J. 245, 185-189

Chapter 4.

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Nicotine Binding Sites in Rat Olfactory and Respiratory Epithelia.

Mol. Pharmacol. submitted

## Summary

### Nicotine as an Odorant

The results suggest that nicotine vapour stimulates an *in vitro* olfactory preparation in three strains of rat and two strains of mouse, in a manner similar to known odorants. Preliminary experiments also suggest that nicotine is an odorant for human subjects.

In the rat, the electro-olfactogram (EOG) produced by nicotine is attenuated by superfusion of the olfactory mucosa with the lectin concanavalin A. This reduction is prevented by  $\alpha$ -methyl-D-mannoside, suggesting that there is a glyco-moiety associated with at least one olfactory receptor responding to nicotine.

A concanavalin A induced change in EOG response with varying odorant concentration for several odorants, including nicotine, can be explained by a single concanavalin A sensitive olfactory receptor with a dissociation constant for odorant binding in the order of 100 nM. The results also show that hydrophilic odorants are poor stimulants for the olfactory epithelium, supporting the hypothesis that the interaction of an odorant with the olfactory receptors involves hydrophobic effects.

Spatial variation in response to four odorants, including nicotine, by the rat olfactory epithelium can be explained by a mosaic of olfactory receptors of various types in the olfactory epithelium. This observation is consistent with current hypotheses of odour quality determination by the olfactory mucosa.

### Nicotine binding sites in olfactory and respiratory epithelia.

Binding studies show that there are sites for  $^3\text{H}(-)$ nicotine in both olfactory and respiratory preparations, though these sites may not be the same in each tissue. The binding parameters for olfactory epithelium are  $K_D=695$  nM and  $B_{max}=8.24$  pmol/mg protein (mean of two experiments at optimal binding conditions). The olfactory epithelium binding sites differ from binding sites for nicotine described elsewhere for brain (e.g.  $K_D$  values from 0.2-60 nM,  $B_{max}$  values from 1-100 fmol/mg protein) and for liver ( $K_D=0.2$  nM,  $B_{max}=5$  fmol/mg protein).

Some of the  $^3\text{H}(-)$ nicotine binding may be to an olfactory receptor, though more conclusive evidence is required to substantiate this.

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## Layout of Thesis

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Chapter 2a. Spatial Variation in Response on the Rat Olfactory Turbinates.

Chapter 2b. The Effect of Concanavalin A on the Rat Electro-olfactogram at Varying Odorant Concentrations.

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Chapter 3. Ligand Binding and the Study of Receptors. Use of the Filtration Binding Assay to Study the Binding of  $^3\text{H}(-)\text{nicotine}$  to Olfactory Membranes.

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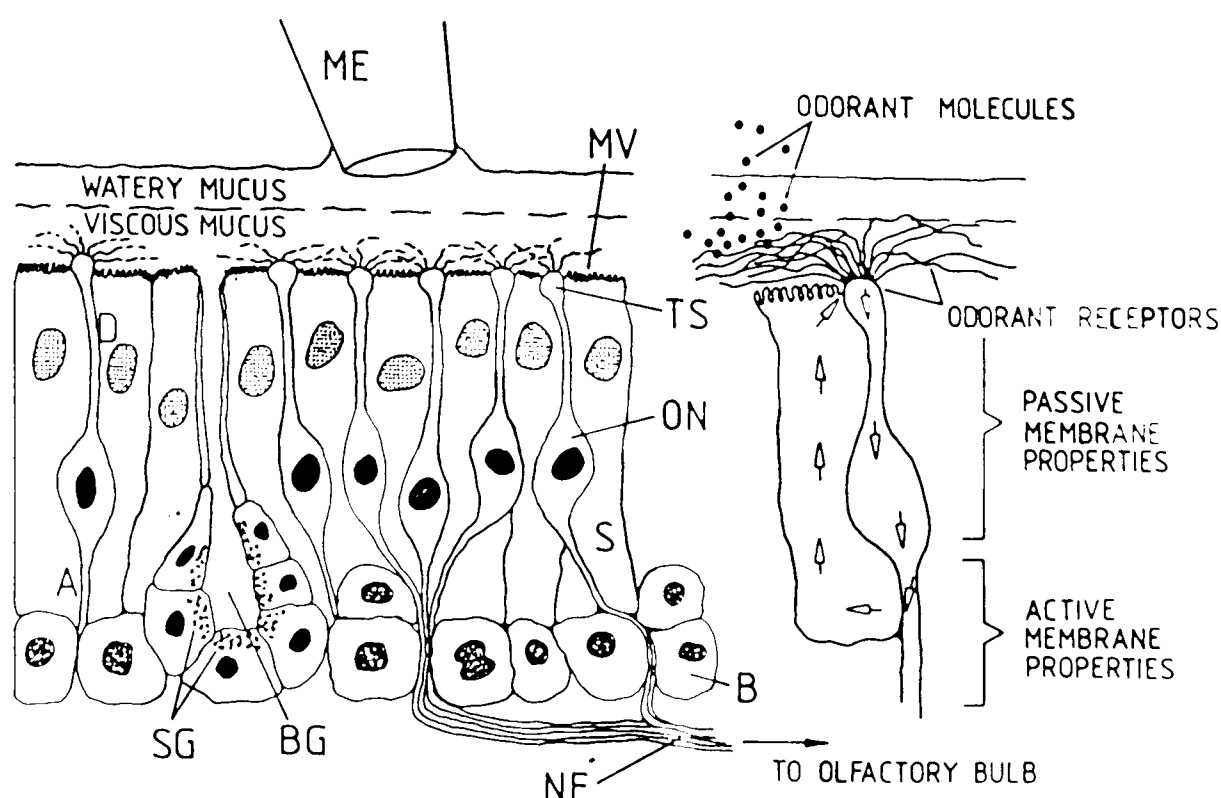
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## General Introduction.

### Identification and isolation of an olfactory receptor

The identification and isolation of an olfactory receptor, i.e. a receptor which links odorant interaction at a specific binding site with transduction pathways, is the prize still awaiting researchers in the field of olfaction. Central to this research effort has been the use of biochemical techniques to identify and characterise parts of the transduction process together with electrophysiological studies to investigate these aspects in the intact tissue or cells. The complexity of the system has made this research challenging. Not only are there olfactory receptors for the "typical" low molecular weight odorants (alcohols, thiols, and fatty acids for example) but also for pheromones (e.g. androstenone in the sow) and for other behaviourally important compounds. A recent report has shown that antigens of the major histocompatibility complex, when degraded in the urine, may be important olfactory cues in the rat (Singh *et al.*, 1987). In addition, olfactory receptor heterogeneity and interaction of an odorant with more than one receptor type are known characteristics of the olfactory process (Polak, 1973; Lancet, 1986; Getchell, 1986). Non-olfactory receptors of the nasal cavity such as the vomeronasal organ and the trigeminal nerve are also important in the overall function of the nasal chemosensory systems (Keverne *et al.*, 1986).

Figure 1. Schematic Diagram of the Cells of Vertebrate Olfactory Epithelium

ME - microelectrode (for recording EOGs); MV - microvilli; TS - terminal swelling;

ON - olfactory neuron; S - supporting cell; B - basal cell; NF - nerve fibres; A - axon;

D - dendrite; BG - Bowman's gland; SG - secretory granules;

The arrows on the right hand diagram show the direction of ion currentflow associated with sensory transduction. The olfactory epithelium is typically 100-200 $\mu$ m thick, each olfactory neuron cell body is 5-8 $\mu$ m across and their dendrites are about 2 $\mu$ m wide. The cilia which radiate from the terminal swelling are about 0.25 $\mu$ m wide at the proximal end, narrowing to only 0.06 $\mu$ m at the distal end. Estimates of the number of cilia radiating from each neuron varies from 40 up to 100 depending on the species. Olfactory cilia vary from 30-200 $\mu$ m in length, also dependent on the species. The olfactory neuron's axon is about 0.2 $\mu$ m wide.

[redrawn from various sources including Dodd & Squirrell, 1980; Getchell, 1986.]

It is generally accepted that the receptor sites are found on the non-motile cilia which radiate from the olfactory neurons and into the mucus layer covering the epithelium (for a recent electrophysiological study on loss of response to odorants following cilia removal see Adamek *et al.*, 1984). The structure and function of olfactory neurons and other cells of the mucosa have been adequately reviewed elsewhere (Dodd & Squirrell, 1980; Getchell, 1986; Lancet, 1986). A schematic diagram of the cells of the olfactory epithelium is shown in figure 1. Other workers have recently described the preparation of olfactory cilia or cilia membrane preparations for use in biochemical studies, a development which is essential to any effort to isolate an olfactory receptor (Rhein & Cagan, 1980; Chen *et al.*, 1986a; Shirley *et al.*, 1986).

There have been several attempts to identify and isolate an olfactory receptor, predominantly by binding radiolabelled odorants to preparations of olfactory mucosa (Gennings *et al.*, 1977; Dodd & Persaud, 1981; Price, 1978; Fesenko *et al.*, 1979; Rhein & Cagan, 1980; Pelosi *et al.*, 1982; Wood & Dodd, 1984; Pevsner *et al.*, 1985). These studies have identified odorant binding sites but it is unclear in many cases whether the observed binding is to an olfactory receptor and/or to another component of the olfactory mucosa (e.g. see Pevsner *et al.*, 1986). For example, odorants may bind to detoxification enzymes which are present in rat olfactory epithelium (Hadley & Dahl, 1982; Bond, 1983; Reed *et al.*, 1986; Jenner & Dodd, 1988). Another approach has been to identify a component of olfactory cilia with

characteristics expected for an olfactory receptor i.e. ciliary enrichment, transmembrane orientation and relevant concentration in the olfactory membrane. The glycoprotein (gp95) identified by Chen *et al.* (1986b) may be an important component of the sensory process.

These approaches are by no means exhaustive. Research into specific anosmias (the inability to detect a specific odorant) may provide useful information on the nature of olfactory receptors (Amoore, 1967). It has been suggested that there may be up to 30 classes of anosmia though it is difficult to estimate the number of different types of olfactory receptor which might be involved. Identification of anosmic strains of mice should facilitate study into the molecular basis of anosmia (Price, 1977; Wysocki *et al.*, 1977). Evidence also suggests that specific anosmias are determined genetically (Wysocki & Beauchamp, 1984), raising the possibility that anosmia is a result of a defective gene or gene product (most likely to be one type of olfactory receptor).

Chemical modification of olfactory receptors *in vivo* and in electrophysiological studies has also been used to study the nature of olfactory receptors. The strategy in these experiments is to treat the olfactory mucosa with a reagent in order to selectively alter the response to odorants. Treatment of the olfactory mucosa with the reagent, N-ethylmaleimide (Getchell & Gesteland, 1972), with mersalyl (Menevse *et al.*, 1978), and by enzymatic iodination (Shirley *et al.*, 1983b) has shown



selective effects on the response to odorants. Protection from the effects of these reagents by odorants suggests that the reagents are acting at or close to the olfactory receptors. Affinity labelling of the olfactory mucosa with odorants such as ethyl bromoacetate *in vitro* (Persaud *et al.*, 1981) and ethyl-n-butyrate *in vivo* (Mason & Morton, 1984; Mason *et al.*, 1984) and photoaffinity labelling of the olfactory epithelium with light-activated odorants (Menevse *et al.*, 1977) has shown selective effects on the response to odorants. The most in depth investigation of olfactory receptors using the chemical modification approach has been done in this laboratory. The lectin concanavalin A has been shown to modify the response of the olfactory mucosa to odorants in the rat (Shirley *et al.*, 1983a) and in the frog (Wood *et al.*, 1983). Over 100 odorants have been investigated in the rat, showing that the concanavalin A effect is strongest for 4 carbon to 6 carbon alkyl compounds, in particular for short chain fatty acids of which 1-pentanoic acid is the best example studied (Shirley *et al.*, 1987b). The concentration-response profile for the concanavalin A sensitive olfactory receptor has also been studied (Shirley *et al.*, 1987a and Chapter 2). The relationship between the results from chemical modification experiments and events at the molecular level has been discussed in detail elsewhere (Shirley *et al.*, 1987b).

To facilitate the isolation of an olfactory receptor, researchers have investigated the second messenger pathways of olfactory transduction, the strategy being that any prospective olfactory receptor which may be isolated can be tested in reconstitution experiments with components of the

transduction process. In this way the criteria for an olfactory receptor (e.g. as described by Lancet, 1986) should be fulfilled. To this end, work in this laboratory on the rat olfactory adenylate cyclase (see below) is progressing well (Shirley *et al.*, 1986; Shirley *et al.*, 1987<sub>c,d</sub>).

### Recent Advances in Olfactory Biochemistry

In recent years the research effort into understanding olfactory mechanisms has become increasingly multi-disciplinary and has led to many interesting advances. For example, messenger RNA for the olfactory marker protein has been isolated (Rogers *et al.*, 1985) and the amino acid sequence of the protein itself has now been determined (Sydor *et al.*, 1986). The function of this cytosolic, olfactory neuron-specific protein is still unknown. Perhaps most encouraging has been the advances in identifying olfactory transduction mechanisms.

An odorant-modulated adenylate cyclase enzyme cascade involving G-proteins is known to be present in olfactory cilia of both rat and frog (Kurihara & Koyama, 1972; Menevse *et al.*, 1977; Pace *et al.*, 1985; Sklar *et al.*, 1986; Shirley *et al.*, 1986; Pace & Lancet, 1986; Anholt *et al.* 1987). This, together with evidence from patch clamp studies on isolated olfactory neurons identifying ion channels (Nakamura & Gold, 1987; Labarca *et al.*, 1987), evidence for phosphorylation of specific membrane proteins following stimulation of the olfactory adenylate cyclase (Heldman & Lancet, 1986) and

the presence of a family of olfactory phosphodiesterases (K. Dickinson, S.G. Shirley & G.H. Dodd, unpublished results, this laboratory), suggests that a probable transduction pathway has been identified. The similarities of this transduction process with that identified in vision have been noted (Lancet, 1986; Nakamura & Gold, 1987).

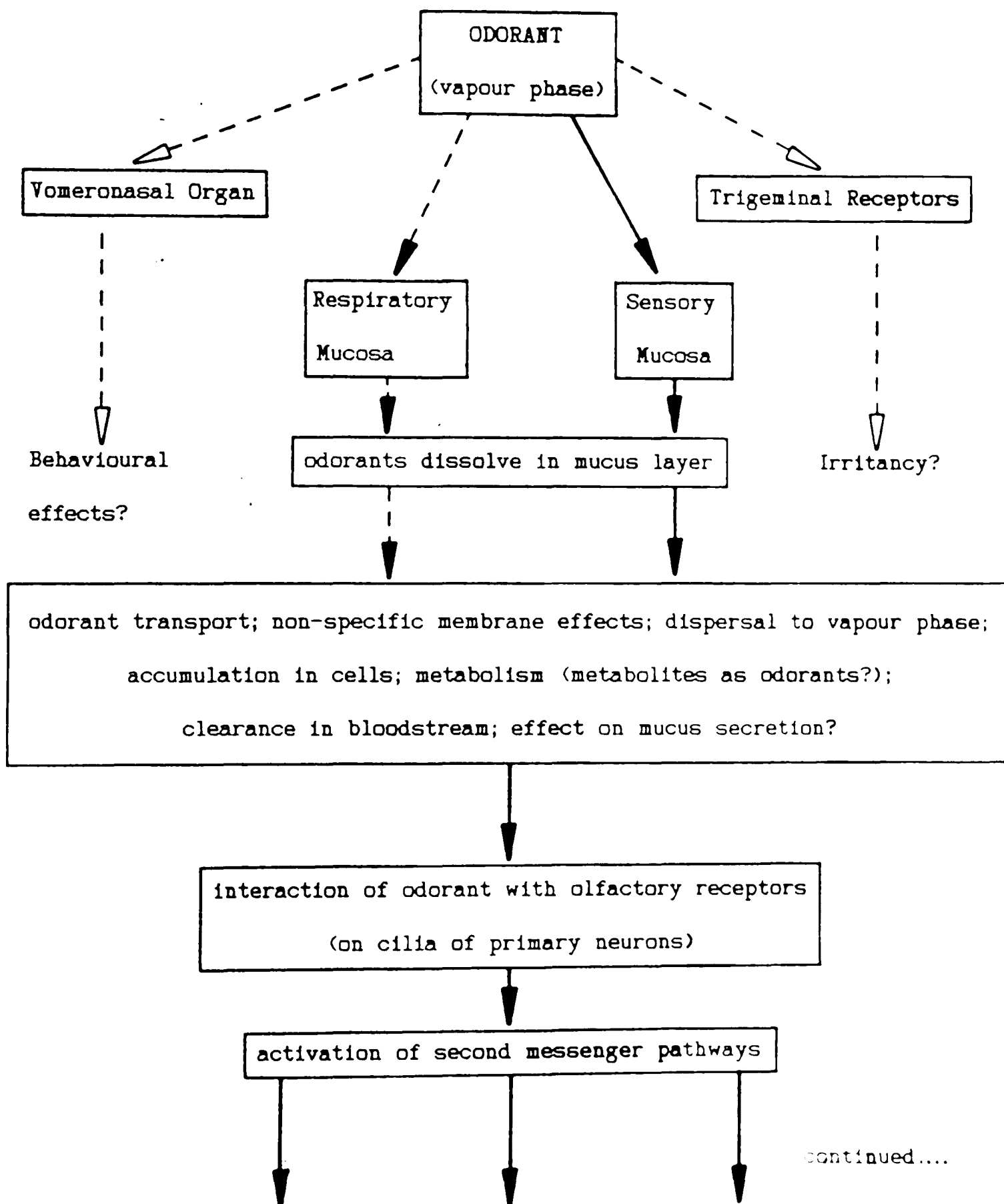
However, it is also known that some odorants are poor agonists for the olfactory adenylate cyclase (Sklar et al., 1986) suggesting that additional olfactory transduction mechanisms have still to be identified. One candidate for this is the phosphoinositol second messenger system (e.g. Hokin, 1985; Irvine, 1987) which involves both cyclic nucleotides and G-proteins (Michell & Kirk, 1986). Phosphoinositide metabolism has been measured in fish olfactory cilia (Huque & Bruch, 1986) although other recent evidence shows that phospholipase C is found in both sensory (olfactory) and non-sensory (respiratory) cilia preparations of the frog (Anholt et al., 1987). Thus, it is unclear whether phosphoinositide metabolism is linked to a specific olfactory transduction mechanism. Work in this laboratory (Y. Russell, M. Wood, R. Aujla & G.H. Dodd, unpublished results, this laboratory) may help clarify the role of phospholipids in olfactory transduction.

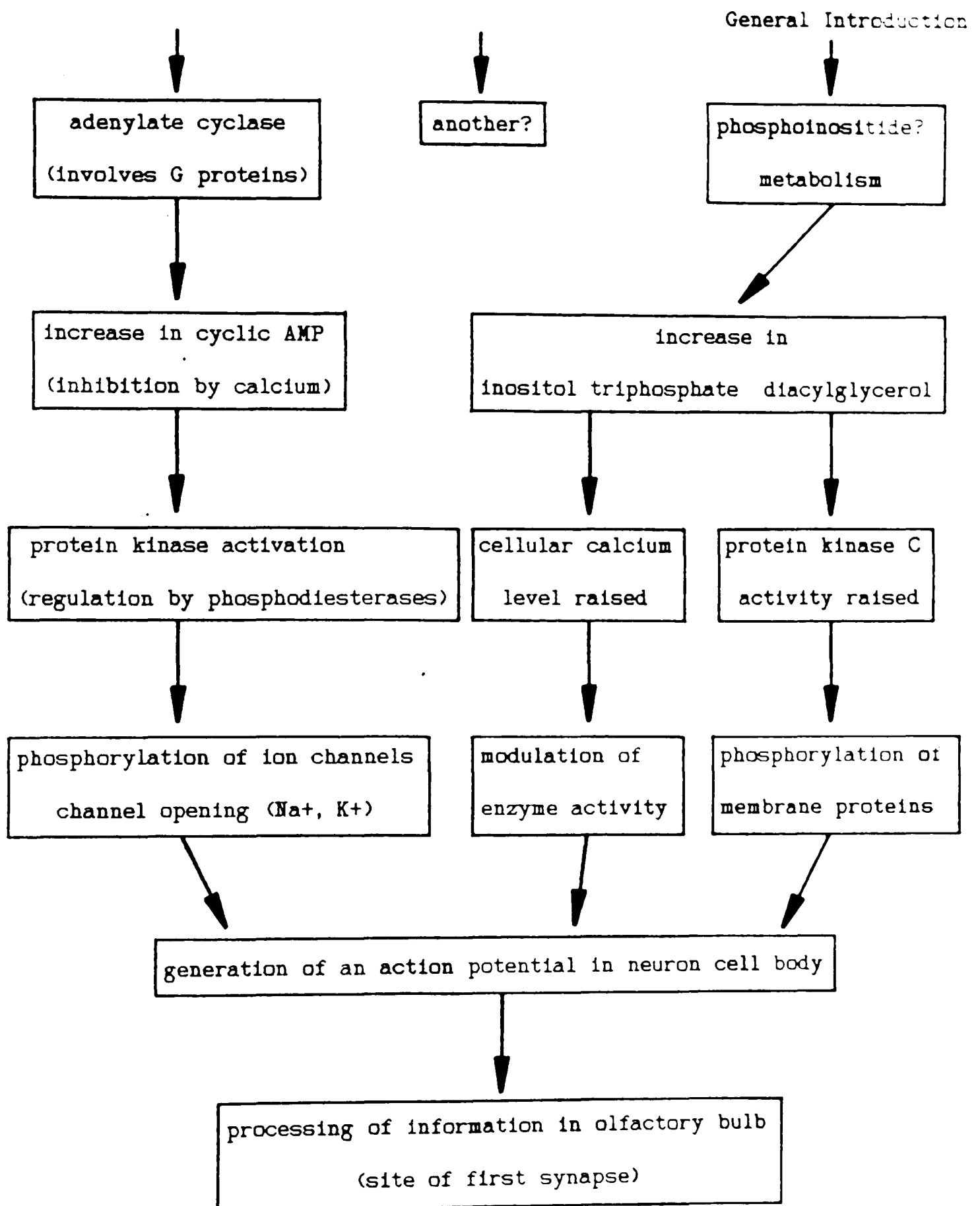
Advances in our knowledge of olfaction are not limited to the transduction mechanisms. Studies have shown that there is homology between olfactory proteins and proteins from other sources. The odorant binding protein, OBP, first identified by its ability to bind

2-isobutyl-3-methoxypyrazine (Pelosi *et al.*, 1982; Wood & Dodd, 1984; Pevsner *et al.*, 1985; Pevsner *et al.*, 1986) is a soluble protein localised to olfactory mucus and is secreted from the Bowman's glands of the olfactory epithelium. The OBP has been suggested to play an important role in odorant transport. Structural homology between the pyrazine binding protein itself and a family of urinary proteins of unknown function has been demonstrated (Cavaggioni *et al.*, 1987). It was suggested that these proteins play a role in odorant transport in urine which may have important behavioural effects (see Singh *et al.*, 1987 in opening paragraph also). Using molecular cloning techniques on a complementary DNA library from olfactory tissue of the frog, it has been shown that an olfactory specific messenger RNA which is localised to the cells of the Bowman's glands, codes for a protein with amino acid sequence homology to serum transport proteins and a retinol binding protein (Lee *et al.*, 1987).

A summary of the events leading to odorant detection and signal transduction by the olfactory neurons is shown in table 1.

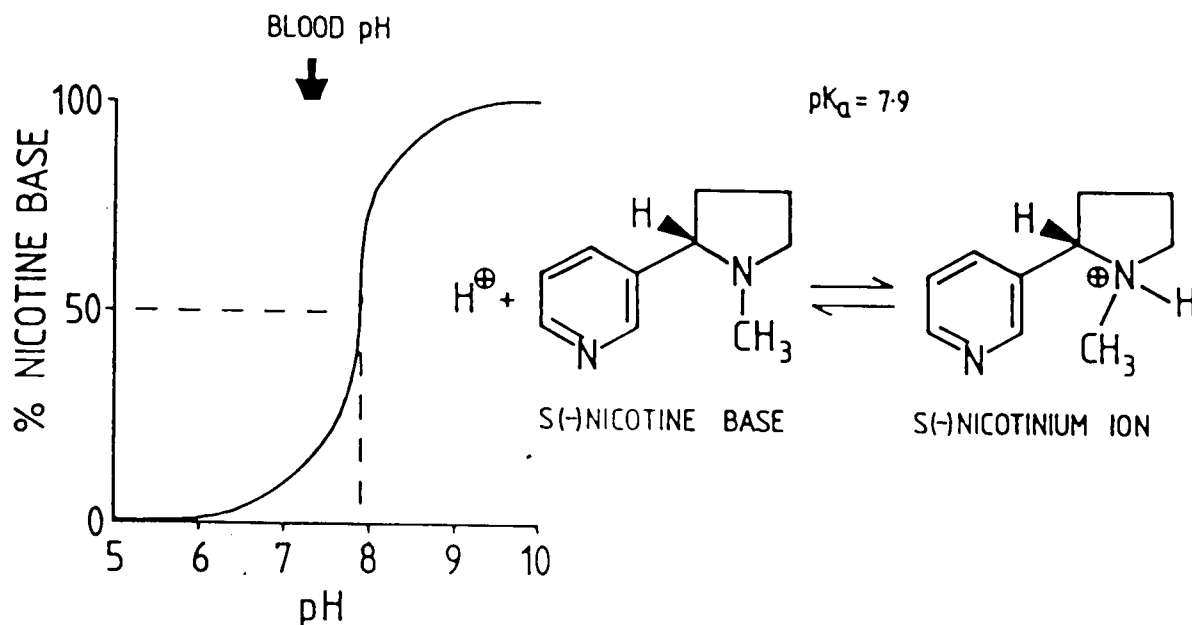
Table 1. Olfactory Stimulus Reception and Transduction.





## The Pharmacology and Biochemistry of Nicotine

Figure 2. Nicotine Structure and pKa



[redrawn from Mangan & Golding, 1984]

Nicotine (figure 2), an alkaloid found in tobacco plants and hence in tobacco products, was first isolated in the early 1800's. The chemistry of nicotine has been widely studied (e.g. Jackson, 1941; Seeman, 1984), principally because of its potent pharmacological effects as an agonist for the acetylcholine receptor (e.g. Taylor, 1980). Much is known about the nicotinic acetylcholine receptor (Wan & Lindstrom, 1984) including details on neurotoxin binding sites (Wonnacott *et al.*, 1982) subunit structure (Criado *et al.*, 1985) and functional arrangement within the membrane

(Hamilton *et al.*, 1985; Maelicke, 1987). Nicotine is also well studied since it is probably the most important component of tobacco smoke, causing addiction to the smoking habit (Henningfield *et al.*, 1985). The effects of nicotine on smokers are too numerous and complex to cover adequately here but have been reviewed excellently elsewhere (e.g. Dawson & Vestal, 1982; Hall, 1982; Balfour, 1982; Mangan & Golding, 1984; Benowitz, 1986). The action of nicotine on the cardiovascular system (e.g. Fenton & Dobson, 1985; Benowitz, 1986), in particular on that of smokers with hidden or inherited heart defects, is thought to be the most harmful of nicotine's effects on the body.

The biochemistry of nicotine is equally complex and fascinating. Radiolabelled nicotine has been used in receptor binding studies to identify binding sites in several tissues. Possibly of most importance is the binding of nicotine to brain membrane preparations of the rat (Abood *et al.*, 1985b; Romano & Goldstein, 1980; Sloan *et al.*, 1984; Lippiello & Fernandes, 1986) mouse (Marks & Collins, 1982; Seršen *et al.*, 1981) and man (Shimohama *et al.*, 1985). This binding site for nicotine is stereoselective in favour of the (-)-isomer (for details see table 2) which is also the case in pharmacological tests of the potency of the two isomers of nicotine (for early work see Barlow & Hamilton, 1965). Evidence suggests that the neuronal nicotine binding site is not the same as the neuromuscular nor ganglionic nicotinic acetylcholine receptor (e.g. Marks *et al.*, 1986; Wonnacott, 1986; Kemp & Morley, 1986; Collins *et al.*, 1986) and it is still a point of debate whether the brain binding site is cholinergic



(e.g. Abood *et al.*, 1985 c.f. Marks & Collins, 1982) and how many nicotine binding sites in brain there are (e.g. Sloan *et al.*, 1984). Other workers have shown that nicotine influences the metabolism of proteins in rat brain (Sershen & Lajtha, 1979; Sershen *et al.*, 1982) and high concentrations of nicotine (>1 mM) have been shown to stimulate protein synthesis in mouse tissue-culture cells (Hunt & Kelley, 1984). The correlation of all these results with those from behavioural studies (Marks *et al.*, 1985a,b; Henningfield *et al.*, 1985) will assist in understanding the complex effects of nicotine on the brain.

Nicotine also binds to non-cholinergic sites (selective for the (+)isomer) on human leucocyte membranes (Davies *et al.*, 1982; Hoss *et al.*, 1986), the significance of which is still unclear, although nicotine is known to be chemotactic for neutrophils (Totti *et al.*, 1984) and may reduce the anti-microbial effectiveness of human polymorphonuclear leucocytes (Sasagawa *et al.*, 1985). There is a non-cholinergic binding site for nicotine on rat hepatocytes and hepatocyte membranes (Abood *et al.*, 1985a) which may be linked to a transport mechanism and/or metabolism of the alkaloid by liver enzymes. Metabolism of nicotine in the liver to cotinine, (see appendix A) for excretion in urine, is the major pathway by which nicotine is eliminated from the body (Mangan & Golding, 1984). Weak binding of nicotine to human plasma lipoproteins (Maliwal & Guthrie, 1981a) and human serum albumin (Maliwal & Guthrie, 1981b) has also been demonstrated. Another effect of interest is the increase in mucociliary

activity of the rabbit maxillary sinus following introduction of nicotine into the blood supply (Lindberg *et al.*, 1985).

The characteristics of nicotine binding to various tissues are summarised in table 2 from a selection of references.

Table 2. Nicotine Binding Study Results.

author (assay)	isomer	K <sub>D</sub> (nM)	B <sub>max</sub> (fmol/mg)	stereoselectivity
<hr/>				
Rat Brain				
Romano & Goldstein	(±)	28	3.2	(-) by 60 fold
1980 (F)		460	10.4	
Vincek <i>et al.</i> pH 8.4	(-)	63		(-) by 3 fold
1981 (C) pH 8.4	(+)	220		
Aboud <i>et al.</i>	(-)	0.2	5	(-) by 3 fold
1983 (C)		2	29	
Costa & Murphy	(±)	23.7	76	(-) by 60 fold
1983 (F)		590	646	
Sloan <i>et al.</i>	(±)	14	1	(-) by 80 fold
1984 (F)		1146	23	
Clarke <i>et al.</i>	(±)	3.5	7	(-) by 17 fold
1984 (A)				

# General Introduction

Abood <i>et al.</i>	(-)	0.2	5	(-) by 10 fold
1985b (C)		3	15	
(as above) pH 8.4	(+)	7	15	
Benwell & Balfour	(±)	2.9	30	(-) 20-90 fold
1985 (F)		11900	20000	
Wonnacott	(±)	49	71	(-) by 88 fold
1986 (F)				
Lippiello & Fernandes	(-)	2	200	(-) by 60 fold
1986 (F)				

## Mouse Brain

Sershen <i>et al.</i>	(±)	60	50	(-) if any
1981 (F)		20000	9800	
Marks & Collins	(±)	59	88	(-) 30-40 fold
1982 (F)				

## Human Brain

Shimohama <i>et al.</i>	(-)	8.1	36	(-) by 40 fold
1985 (F)		86	90	

## Human Leucocytes

Davies <i>et al.</i>	(±)	36	87000 sites/cell	(+) by 30 fold
1982 (C)				
Hoss <i>et al.</i>	(±)	17	65300 sites/cell	
1986 (C)	(-)	8	48700 sites/cell	(+) by 100 fold

Rat Hepatocytes					
Aboud <i>et al.</i>	pH 8.5	(-)	0.2	5	
1985a (C)	pH 8.5	(+)	3	4	
(as above)	pH 8.5	(+)(-)	4	50	none

values were determined at pH 7.4-7.7 unless stated otherwise.

C = centrifugation assay; F = filtration assay; A = autoradiography

Nicotine and Olfaction

My interest in nicotine arose initially from a study by Hedlund & Shepherd (1983) which showed that a (muscarinic) cholinergic binding site could be detected in the olfactory mucosa of the salamander. At this time, I was considering tobacco smoke in relation to olfaction and I was interested to discover whether nicotine had any documented effects on the olfactory mucosa. Substituted pyridines are known to be important flavour compounds in many food products such as tea and coffee (Vernin, 1982) and nicotine is thought to be a flavour compound in tobacco (Enzell, 1981; Vernin, 1982). Nicotine was described in the literature as a volatile, colourless liquid (Jackson, 1941; Mangan & Golding, 1984) which was said to "turn brown and acquire the odour typical of tobacco" on exposure to the air (Mangan & Golding, 1984). It was also suggested that nicotine may have important effects on many sensory processes which would be secondary reinforcement factors for the smoking habit (Mangan & Golding, 1984).

Other than these references, I could find no detailed evidence to suggest that nicotine was a stimulant for the olfactory receptors of the nasal epithelium. It has recently been shown that nicotine stimulates the trigeminal receptors of the nasal cavity (Silver & Walker, 1987).

This was a timely opportunity to investigate the effect on olfactory mucosa of a potent pharmacological compound with well documented physiological and biochemical properties. My strategy was to determine whether nicotine vapour stimulated the rat olfactory preparation to produce an electro-olfactogram (EOG), and if so, to characterise this EOG using established procedures. Following this I intended to investigate the binding of radiolabelled nicotine to a membrane preparation from rat olfactory epithelium, which was known to contain the odorant-modulated adenylate cyclase (Shirley *et al.*, 1986). I hoped that any binding measured might be related to an olfactory receptor. Ideally, this work would also show any effects nicotine may have on rat olfactory epithelium which may be related to human olfaction. Most of us are exposed to nicotine and possible harmful effects (Hoffmann *et al.*, 1985) through active and/or passive smoking in many social situations (Williams *et al.*, 1985).

## Section 1.

### Chapter 1.

#### Evidence for an Olfactory Receptor which responds to Nicotine -Nicotine as an odorant.

##### Introduction

The biochemical properties of nicotine and in particular its effects as an agonist for the nicotinic acetylcholine receptor, have been extensively reviewed in the literature (see General Introduction and table 2). Nicotine is known to be the primary satisfaction factor for tobacco and to influence the flavour of the smoke considerably (Enzell, 1981). It occurs at high levels in tobacco products, typically 1.8 mg of nicotine per cigarette (Mangan & Golding, 1984). Considering the latter and the amount of work carried out with nicotine, it is surprising that there are no detailed studies reported on nicotine as a stimulant for the olfactory epithelium. Recent evidence has shown that nicotine stimulates the trigeminal receptors of nasal mucosa (Silver & Walker, 1987).

The saturated vapour phase concentration above pure nicotine is calculated to be 4 micromolar at 20°C, which is likely to produce perceivable concentrations in the olfactory mucosa (see appendix A). It

has been noted that nicotine evaporates appreciably when exposed to room air (Jackson, 1941).

The aim of this work is to describe the action of nicotine on olfactory epithelium using the electro-olfactogram to measure stimulation. The results are compared with the stimulatory properties of some known odorants. I have also used established chemical modification methods for olfactory epithelium (Shirley *et al.*, 1983a; Shirley *et al.*, 1987b) to modify the response of the olfactory receptor(s) for nicotine.

## Materials and Methods

### Chemicals

i-Pentyl acetate (i-amyl acetate), 97%, (Aldrich Chemical Co., U.K.), i-pentanoic acid, 98%, (Fluka AG., W. Ger.) and cineole, 99%, (BDH Chemicals Ltd., U.K.), were used in the experiments without further purification. (S)(-)-nicotine, concanavalin A type IV and  $\alpha$ -methyl-D-mannoside grade III were from Sigma Chemical Co., U.K. Male Wistar, Sprague Dawley and Lister Hooded rats (200-250g) and male Balb/c and MF1/Ola mice (7 weeks old) were from Harlan Olac Ltd., U.K. All other reagents used were of analytical grade.

The nicotine was redistilled under reduced pressure to 99.9% purity (major impurity < 0.1%) and stored under nitrogen at -20°C and in the dark. After 15 months storage under these conditions, the nicotine had discoloured but was still 99.7% pure. The purity was determined by capillary gas liquid chromatography. For further details see appendix A.

Redistilled nicotine was diluted to 20% (v/v) in paraffin (Fisons water white liquid paraffin, specific gravity 0.83-0.86) and stored in the dark under nitrogen at room temperature before use. A fresh dilution was made every five days, or earlier if the solution had discoloured. Dilutions in paraffin of 1-pentyl acetate, 1-pentanoic acid and of cineole were also used in the experiments. Passage of clean dry air across the surface of 2 ml of this odorant solution produced vapour which was diluted with filtered, humidified air and allowed to equilibrate in the apparatus before use. The air used in all EOG studies was filtered under pressure through a column of charcoal, molecular sieve and silica gel prior to use. The 2 ml aliquots of diluted odorant were kept at constant temperature (15°C) and were used for 24 hours, after which the solutions were changed. In the case of nicotine, the odorant container and stock solutions were replaced at the first sign of discolouration. Preliminary experiments indicated that the 2 ml aliquot was sufficient for a 24 hour period. Regular checks were made to determine whether or not any vapour from the paraffin alone elicited a response on the rat half head preparation. On the few occasions that a response was detected, the paraffin and all stock solutions were replaced.

The odorant application system was essentially as used previously (Shirley *et al.*, 1983; Shirley *et al.*, 1987b) and as described in detail elsewhere (Shirley, 1987).



### Composition of Locke's (mammalian heart) Ringer solution

Solution A (stored at room temperature), per 1000 ml:

90g NaCl / 4.2g KCl / 3.2g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

Solution B (refrigerated), per 100 ml:

1g  $\text{NaHCO}_3$  / 10g glucose / 18mg ascorbic acid

Ringer solution: 100 ml A + 20 ml B made up to 1000ml in  $\text{H}_2\text{O}$

gassed with 5%  $\text{CO}_2$  / 95%  $\text{O}_2$  prior to use

### Recording electro-olfactograms (EOGs)

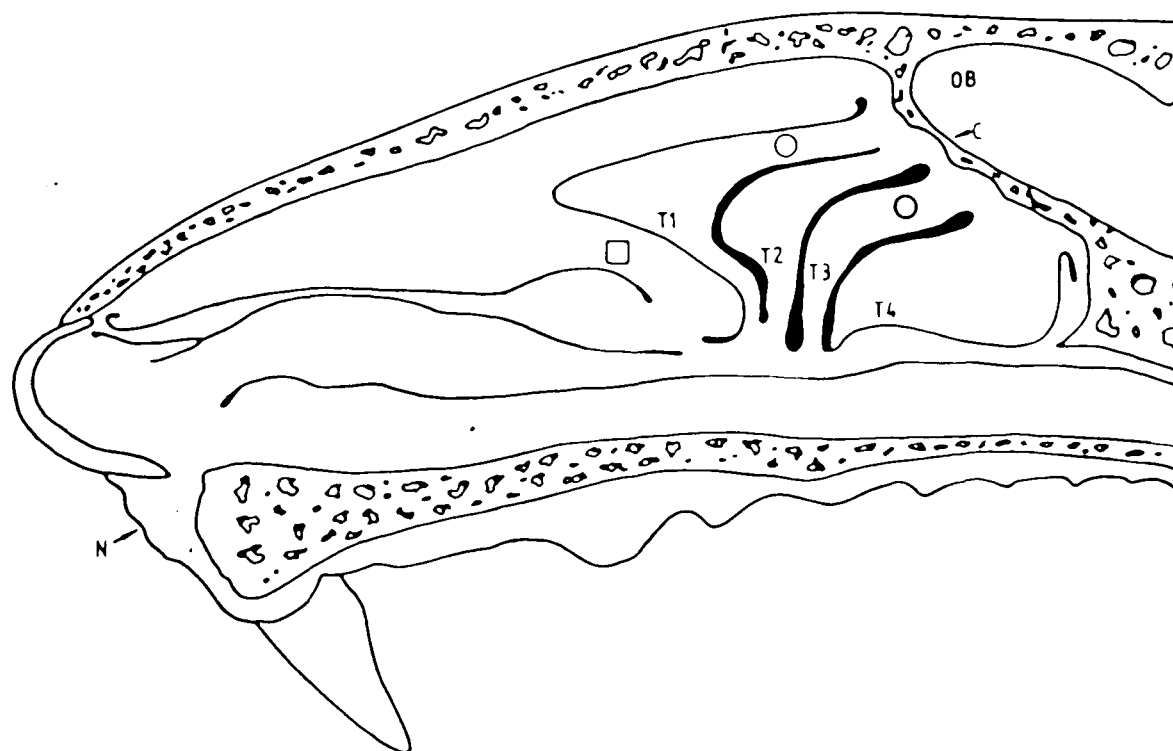
The procedures for recording EOGs from the olfactory epithelium and for concanavalin A modification were essentially as described elsewhere (Shirley *et al.*, 1983a; Shirley *et al.*, 1987b).

The animal was stunned and killed by cervical dislocation. Following decapitation, the head was cut in sagittal section and the exposed septum was immediately removed, taking care not to touch the underlying olfactory turbinates. The half head was mounted on the cooled head stage (the head temperature was kept below  $17^\circ\text{C}$ ) for a 15 minute superfusion with oxygenated Locke's Ringer solution, (flow rate 2ml/minute) after which the Ringer solution was removed by aspiration. EOGs were recorded after a 15 minute rest period and after a steady baseline had been attained.

For the dose-response relationship and concanavalin A experiments, the odorants were presented to the epithelium as a 1 second vapour pulse, followed by a 1 minute recovery period. Duplicate presentations of each test odorant were made to the epithelium during any one recording period.

Recordings were taken from the third turbinate, from a region denoted by the circle on T3 of figure 3.

Figure 3. Schematic Diagram of a Rat Head Following Saggital Sectioning and Removal of the Septum



OB - olfactory bulb; C - cribriform plate; N - naris; T1, T2, T3, T4 - exposed surfaces of the olfactory turbinates. The regions from which recordings were taken are denoted by circles on olfactory turbinates and by a square on respiratory epithelium. The anterior to posterior measurement for the olfactory turbinates was typically 1 cm in an adult male rat.

A standard odorant, 1-pentyl acetate, at a fixed concentration, was presented to the epithelium at regular intervals (every third or fourth

application). 1-Pentyl acetate has been widely used as a standard or reference odorant by workers in olfaction (Shirley *et al.*, 1983a,b; Shirley *et al.*, 1987a,b; Wood *et al.*, 1983).

The response measured in these experiments was the amplitude of the initial EOG peak. The normalised EOG response was obtained on dividing the test odour EOG at time  $x$  by the standard odour EOG, also at time  $x$  (calculated by interpolating from neighbouring presentations of the standard odorant). The mean of this value for each presentation of the same odorant is the value  $\langle A \rangle$ . Use of the  $\langle A \rangle$  value enabled us to take account of variation in EOG amplitude between rats.

#### Concanavalin A treatment

The lectin concanavalin A (McKenzie *et al.*, 1972; Gunther *et al.*, 1973) has been widely used to investigate molecular properties of membranes and proteins (for a review on lectin-membrane interactions see Grant & Peters, 1984). One example is the nicotinic acetylcholine receptor whose function can be inhibited by the lectin (Messing *et al.*, 1984). Lectins are useful since they bind specifically to the sugar residues of glycolipids and glycoproteins, and the effect can be prevented by including free sugar residues in the treatment. Concanavalin A and  $\alpha$ -methyl-D-mannoside have been used to study the electrophysiological response of olfactory receptors (Shirley *et al.*, 1987b).

After the initial odorant sequence had been applied to the epithelium, the electrode was lifted from the tissue. This was followed by a 5 minute rinse of the epithelium with concanavalin A (0.5mg/ml, 2ml/min) in

oxygenated Ringer solution. A 10 minute Ringer-only rinse, washed unreacted concanavalin A from the tissue, after which excess liquid was aspirated from the tissue as before. The electrode was then lowered on to the tissue at the same position and a rest period of at least 15 minutes was allowed before an identical sequence of the test odorants was applied. In control experiments concanavalin A was absent from the procedure.

A measure of the EOG survival,  $\langle L \rangle$ , was determined by dividing the mean EOG (in mV) for the standard odorant after treatment, by the mean standard odorant EOG before treatment. Thus, 100% survival of the EOG for the standard odorant during the experiment gave an  $\langle L \rangle$  value of 1. Results were not used from 2 rats which showed unusually low  $\langle L \rangle$  values of  $< 0.5$ , which indicated either a poor preparation or an excessive dose of concanavalin A.

The parameter  $\langle R \rangle$ , necessary for describing modification of the response by a reagent, is defined as the normalised response after treatment divided by the normalised response from the same odorant before treatment. Thus, an EOG which was unaffected by the treatment had  $\langle R \rangle = 1$ , an odorant whose response was diminished by 50% had  $\langle R \rangle = 0.5$ , under conditions such that the response to the standard odorant was unaffected.

**Summary of parameters used to analyse EOGs:**

$$\langle L \rangle = \frac{\text{mean EOG for standard odorant AFTER treatment}}{\text{mean EOG for standard odorant BEFORE treatment}}$$

$$\langle A \rangle = \text{mean} \frac{\text{EOG TEST odorant}}{\text{EOG STANDARD odorant}}$$

$$\langle R \rangle = \frac{\langle A \rangle \text{ AFTER treatment}}{\langle A \rangle \text{ BEFORE treatment}} \quad (\text{for the same odour})$$

**Effect of  $\alpha$ -methyl-D-mannoside (mannoside)**

In these experiments, the lectin was dissolved in Ringer solution with mannoside at a final concentration of 20 mM. This was applied to the epithelium as described above.

## Calculations

The vapour concentration of odorant presented to the epithelium was calculated from the vapour pressure for the odorant at 15°C, interpolated from standard tables (Weast, 1984; see appendix A also).

The concentration of odorant which reached the olfactory receptors could not be determined. Further information on the composition, production and volume of the mucus and on odorant removal from the mucus are required before such calculations would be possible. Some properties of the mucus have been discussed elsewhere (Getchell *et al.*, 1984). For sparingly soluble odorants it was possible to estimate the concentration of odorant in the mucus from the water to air partition coefficient. An odorant with an air/water partition coefficient of  $>50,000$  would not equilibrate between air and mucus, when presented to the epithelium as a vapour pulse of one second. The estimated mucus concentration in this case was taken as the vapour concentration of odorant presented to the epithelium multiplied by 50,000, since the volume of odorized air passing over the mucus was approximately 50,000 times the volume of the mucus (Shirley, 1987). The vapour concentrations of the odorants used in the experiments described in chapter 1 and estimates of the corresponding mucus concentrations of the odorants are shown in table 3 (see appendix A for calculation of the air/water partition coefficient for nicotine).

Table 3. Concentration of Odorants.

Experiments to which these concentrations apply; \* 10 second pulse of odorant, \*\* response versus concentration of nicotine and \*\*\* concanavalin A treatment. The water/air partition coefficient, W/A, is expressed for all forms of the odorant at pH 7.0 and 15°C. VC - vapour concentration and EMC - estimated mucus concentration of odorant. (A) after a 1 second and (B) after a 10 second continuous presentation of vapour.

Odorant	W/A	-log VC	-log EMC	Experiment
1-pentyl acetate	81	7.12	5.22	***
		7.11	5.20	**
		6.96	5.06	*
cineole	250	6.81	4.41	***
1-pentanoic acid	$4.0 \times 10^6$	6.87	2.17	***
nicotine	$2.2 \times 10^6$	8.74	4.05	**
		8.09	3.39	**
		7.78	3.09	**
		7.48	2.78	***
		7.34	2.64 <sup>a</sup>	*
		7.34	1.64 <sup>b</sup>	*
		7.31	2.61	**
		7.01	2.31	**

## Results

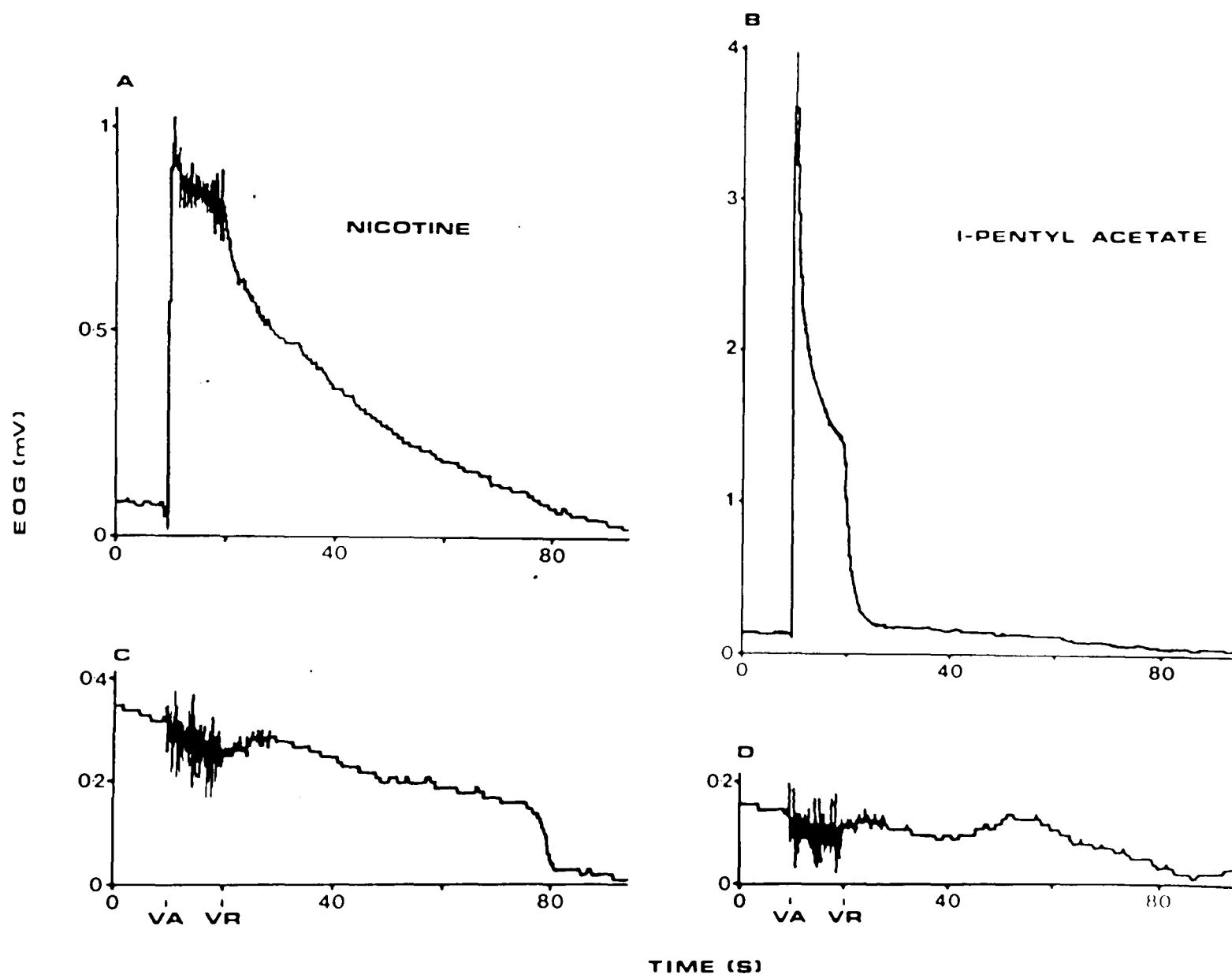
### Nicotine stimulates the olfactory epithelium

If nicotine is to be accepted as an odorant it must stimulate the olfactory epithelium and produce an EOG comparable to the EOGs from known odorants.

Figure 4(a) shows the EOG recorded from the third turbinate following a 10 second pulse of nicotine vapour presented to the Wistar rat olfactory epithelium. The trace shows an initial rapid change in potential of the epithelium on stimulation followed by a reduction in amplitude to a plateau on continued stimulation. When the nicotine vapour was removed, the potential fell immediately towards the baseline value.

The EOG response to a 10 second presentation of nicotine vapour shown in figure 4(a) is comparable to the response of the same preparation to a 10 second 1-pentyl acetate pulse shown in figure 4(b). The removal of odorant from the olfactory epithelium and thus the decay of the EOG response will depend on the solubility of the odorant in the mucus layer and on the  $pK_a$  of the odorant and hence its charge at physiological pH (data for nicotine is given in figure 2). 1-Pentyl acetate was removed from the site of the olfactory response faster than nicotine, as is apparent from the rate of return of the respective EOG traces to baseline value.



Figure 4. EOG Response to Nicotine Vapour

EOG response to a ten second presentation of vapour from (a) and (c) nicotine and (b) and (d) i-pentyl acetate, recorded from the same rat. Traces (a) and (b) were taken from olfactory epithelium and traces (c) and (d) from nasal respiratory epithelium. The concentration of odorants are shown in table 3. The time of vapour application, VA, and of vapour removal, VR, is marked on the x-axis.

The nasal epithelium of the same preparation was then superfused with Ringer solution for three minutes and the electrode moved to a position anterior to the olfactory turbinates as shown by the square in figure 3. Similar 10 second presentations of nicotine and 1-pentyl acetate vapour were made to the preparation, the results of which are shown in figure 4(c) and 4(d) respectively. Neither odorant produced an EOG-like response at this position. The preparation was then superfused with Ringer solution, after which the electrode was placed back onto the third turbinate. EOG responses comparable to those recorded earlier were obtained, indicating that the preparation was still responding to odorants. The preparation was then removed from the head stage and examined under a binocular microscope with incident illumination. The surface of the epithelium in the region denoted by the square in figure 3 had a perceptible motion, suggesting the presence of active respiratory cilia. No movement could be observed on the surface of the epithelium on the third turbinate, from which EOGs to nicotine vapour and 1-pentyl acetate vapour were recorded. These observations were made over 100 minutes after the initial dissection to expose the nasal epithelium of the preparation.

I also recorded this EOG response to nicotine vapour from Lister Hooded and Sprague Dawley rats and from Balb/c and MF1/Ola mice (data not shown).

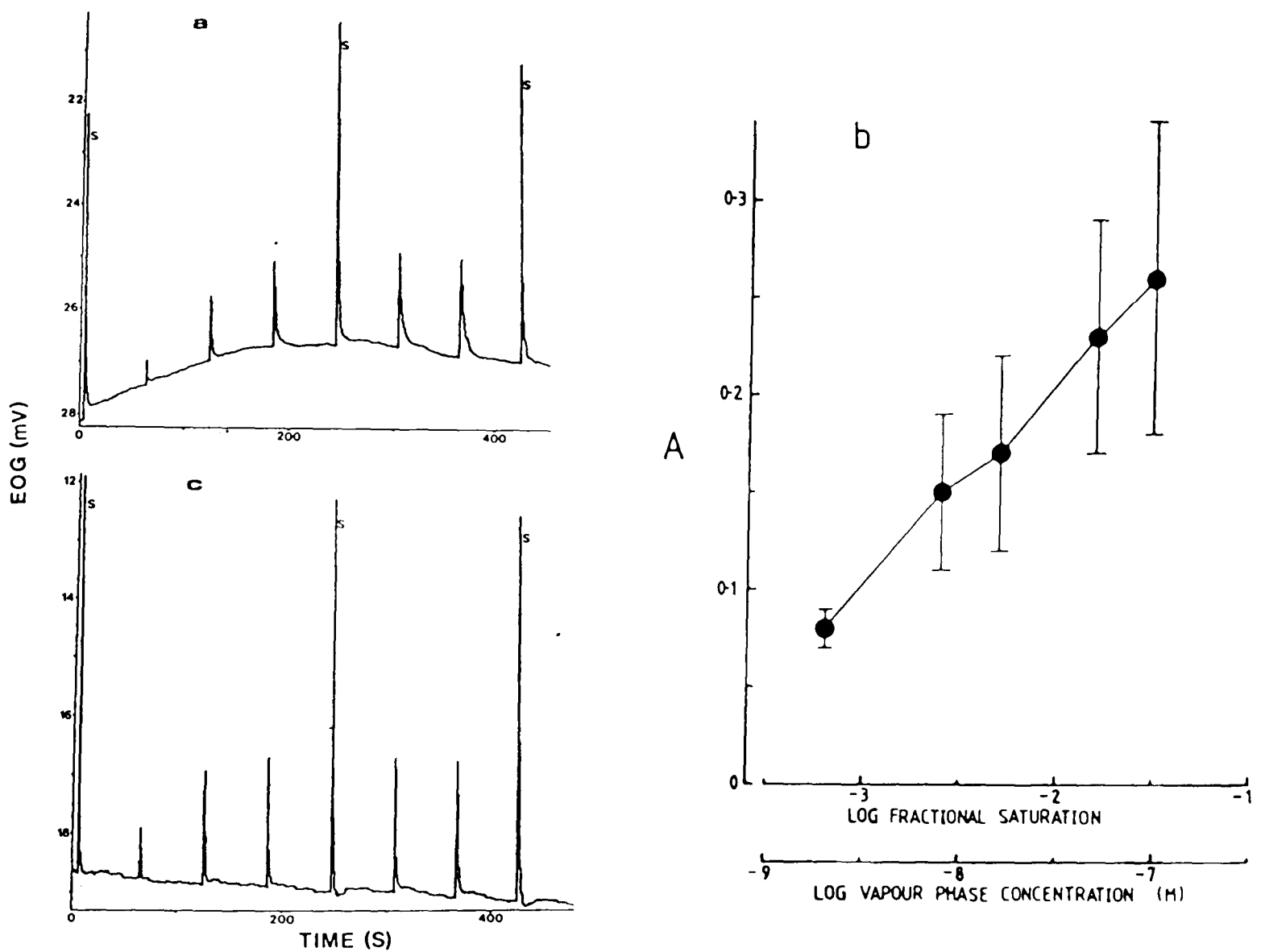
#### **Nicotine EOG dose-response relationship**

Figure 5(a) shows the EOGs to nicotine at various concentrations, recorded from the olfactory epithelium of a Wistar rat in the region

denoted by the circle drawn on T3 of figure 3. Saturation of the response to nicotine, as determined by the  $\langle A \rangle$  value, was not seen over the range of vapour concentrations tested. Higher concentrations were not tested for safety reasons (see appendix A). The results from a group of such experiments are shown in figure 5(b), measured from the third turbinate in the region denoted by the circle drawn on T3 of figure 3.

In some preparations, near saturation of the response to nicotine was seen. An example of this is shown in figure 5(c). Recordings were taken in this case from the region denoted by the circle drawn on T1 of figure 3. Results from two out of the eight cases used in figure 5(b) also showed this pattern of response. It was difficult to determine whether this was best explained by biochemical or experimental factors.

A study on Lister Hooded rats ( $n=5$ ) showed a dose-response relationship to nicotine vapour similar to that seen in the Wistar rat, over the same concentration range (data not shown). Since a large number of variables (such as the unknown concentration of the odorant in the mucus) were involved in this experiment, we did not attempt to calculate a binding constant from the dose-response relationship data. The binding constants of some odorants to olfactory receptors are investigated in chapter 2.

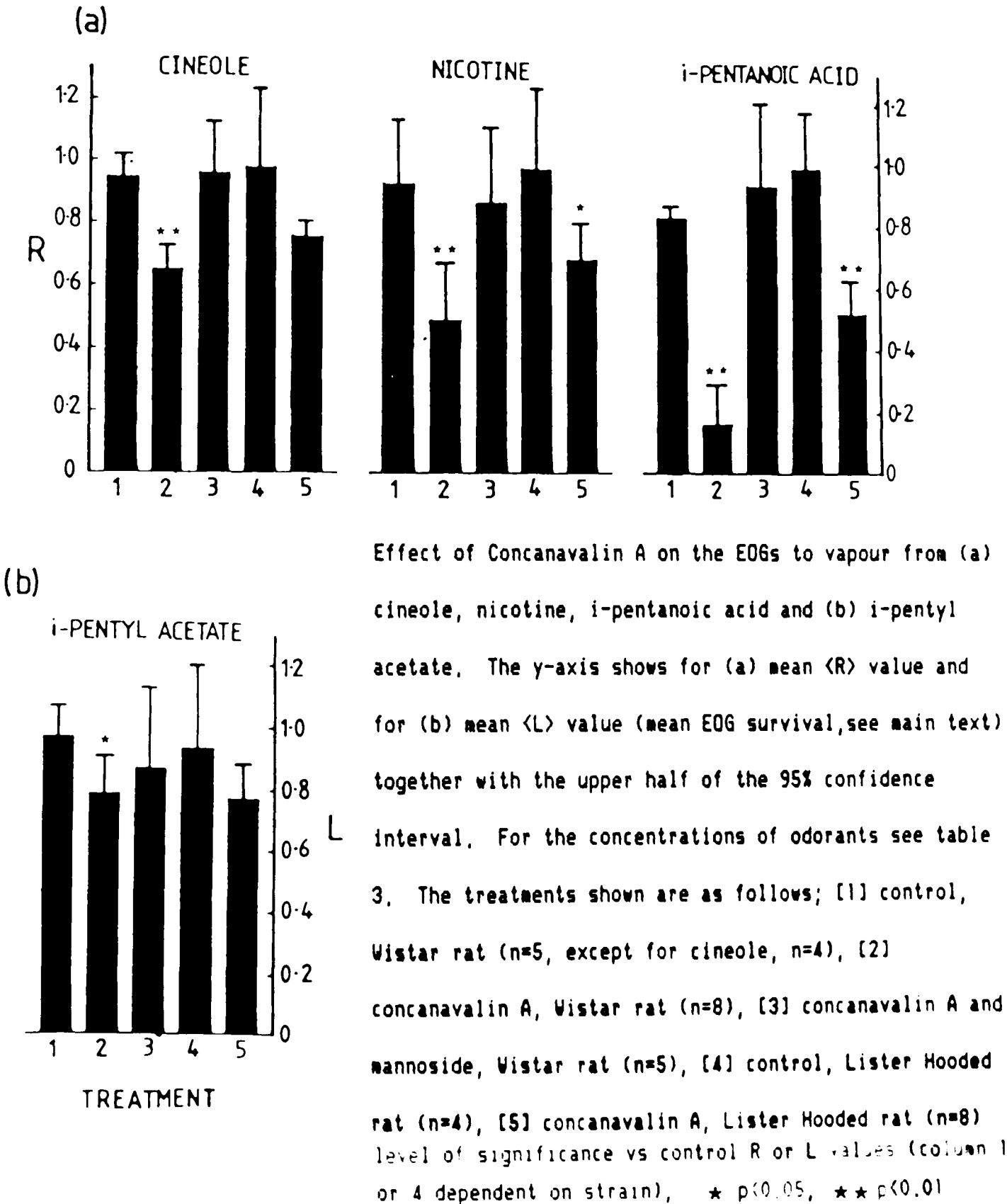
Figure 5. Concentration-Response Relationship for the Nicotine EOG

The recordings, taken from (a) the third turbinate (T3 of figure 3) and (c) the first turbinate (T1), were the result of a one second presentation of odorant and are shown in order of increasing vapour concentration. S shows presentations of the standard odorant, i-pentyl acetate. The concentration of odorants used are shown in table 3. (b) shows a plot of mean  $\langle A \rangle$  value versus log fractional saturation of nicotine, for a sample of studies from the third turbinate only. The error bars show the 95% confidence interval. N is 8, except at the highest nicotine concentration (n is 6). Fractional saturation is a measure of odorant saturation in air at the epithelium surface. Vapour from neat odorant, diluted by a factor of ten before presentation, has a fractional saturation of 0.1.

Concanavalin A inhibition of the nicotine BOG

A concanavalin A modification study was carried out on the rat olfactory response to vapour from nicotine (33nM), cineole (155nM), i-pentanoic acid (134nM) and i-pentyl acetate (75nM).

Figure 6. Effect of Concanavalin A on the Nicotine BOG



The results of a concanavalin A study on the Wistar and Lister Hooded rat are shown in figure 6. The EOGs from cineole, nicotine and i-pentanoic acid were reduced by concanavalin A in both strains of rat. The  $\langle R \rangle$  value for cineole was reduced by 31% and 23%, the nicotine  $\langle R \rangle$  value was reduced by 47% and 30% and the i-pentanoic acid  $\langle R \rangle$  value by 81% and 48% after concanavalin A treatment, in the Wistar and Lister Hooded rats respectively.

Figure 6 also shows the results from the Wistar rat when mannoside was added to the concanavalin A superfusion medium. The results show that the sugar prevented concanavalin A inhibition of the olfactory response to all three test odorants.

The  $\langle L \rangle$  values shown in figure 6(b) represent the "survival" of the EOG response to the standard odorant after each treatment. An analysis of variance revealed that there was no significant difference in  $\langle L \rangle$  between concanavalin A, control, mannoside plus concanavalin A, and strain of rat ( $p > 0.05$ ).  $\langle L \rangle$  reflects effects which the reagent has on all EOGs and any specific effects which it may have on the receptors stimulated by the reference odour. Thus, any non-specific effects on the EOGs were not significantly different between treatments.

#### Effect of cholinergic reagents

In preliminary experiments using a similar protocol as described for the concanavalin A experiments, the effect of cholinergic agonists and antagonists on the nicotine EOG was tested. Acetylcholine, decamethonium, atropine, tetramethylammonium and d-tubocurarine at up to 500  $\mu\text{M}$  in the Ringer superfusion ( $n = 1$  in each case) had no effect on the EOG to

nicotine or four other odorants tested. Nicotine at 5 mM caused a general effect on the EOGs to all odorants tested (<L> values of 0.34 and 0.27, n = 2) whereas at 500  $\mu$ M nicotine did not have such a severe effect and may have caused some selective reduction in the nicotine EOG (as measured by <R>). This was not investigated further.

## Discussion

### Nicotine stimulation of the olfactory epithelium

The EOG, a summated receptor potential, is a useful measure of the initial events of odorant interaction with the olfactory epithelium (Ottoson, 1970). One of the properties of an odorant is the ability to stimulate the olfactory epithelium to produce an EOG.

The results shown in figure 4 suggest that nicotine vapour can stimulate the *in vitro* olfactory preparation to produce an EOG, and as with the known odorant i-pentyl acetate, is unable to stimulate an EOG-like response from nasal respiratory epithelium. These findings suggest the presence of at least one olfactory receptor which responds to nicotine, in the olfactory epithelium of three strains of rat and two strains of mice tested so far.

Nicotine is an unusual odorant in that it has ionizable groups (pKa = 7.9) and will become predominantly charged at physiological pH (90-80% nicotinium ion at pH 7.0-7.4 estimated from Mangan & Golding, 1984; see figure 2 of General Introduction). I assume that the nicotine vapour which

reaches the olfactory epithelium will partition into the mucus layer and from its water/air partition coefficient, I estimate that the nicotine will be concentrated in the mucus by a factor of two millionfold at equilibrium. However, during the course of a typical nicotine vapour pulse in my experiments, equilibrium in the mucus will not be reached. The greater time taken for a soluble odorant (nicotine) to disperse from the olfactory mucus as opposed to a sparingly soluble odorant (i-pentyl acetate), may account for the delay in the potential returning to baseline in figure 4(a) when compared with figure 4(b).

The uncharged nicotine molecule will pass through the membranes of the epithelial cells and as has been suggested for other odorants, may accumulate in the cytoplasm (Getchell *et al.*, 1984). Nicotine is more likely to remain inside the cells than other odorants due to its charge. It is possible that this may produce toxic and other effects which have not been measured in these experiments. In addition, there are several enzyme systems present in the nasal mucosa which could metabolise nicotine (Bond, 1983). For example, nasal cytochrome P-450-dependent monooxygenases have been shown to metabolise nicotine to produce formaldehyde (Dahl & Hadley, 1983). It is possible that metabolites of nicotine may also affect the olfactory system in some way. This is supported by experimental evidence showing accumulation of nicotine or metabolites in the olfactory mucosa (Brittebo & Tjalve, 1983). The metabolism of nicotine by enzymes of the olfactory mucosa is considered in more detail in chapter 4.



### Concanavalin A inhibition of nicotine EOGs

Chemical modification of the olfactory epithelium leads to altered EOG responses and is a possible method for identifying classes of olfactory receptors. Concanavalin A inhibition of the EOG from cineole and especially from 1-pentanoic acid has been observed previously in the Wistar rat (Shirley *et al.*, 1983a; Shirley *et al.*, 1987b) and the results here show a similar effect on nicotine EOGs (figure 6).

Concanavalin A affected the EOGs from the three odorants tested in Wistar and Lister Hooded rats, to different extents. Two-way analysis of variance of the  $\langle R \rangle$  values from concanavalin A treated rats confirmed that there was a difference between the two strains. The results of this analysis can be summarised as follows. First, there was a difference between Wistar and Lister Hooded rats with respect to overall concanavalin A effect ( $p < 0.0005$ ). Secondly, there was a difference between the concanavalin A effect on cineole, nicotine and 1-pentanoic acid ( $p < 0.001$ ) and finally, the differences in the  $\langle R \rangle$  values for the three odorants in the Wistar rat were not significantly different from the differences in the  $\langle R \rangle$  values for the three odorants in the Lister Hooded rat ( $p > 0.05$ ).

The  $\langle R \rangle$  values for all three odorants were reduced to a lesser extent following concanavalin A treatment in the Lister Hooded rat than the Wistar rat. This may be explained by differences between the olfactory mucosa of the two strains with respect to mucus composition, mucus thickness, receptor density and receptor type. These differences may influence the ability of the odorants to stimulate the epithelium (there is no evidence to support this) and the effectiveness of the concanavalin A superfusion.

Consistent with this notion is the observation that ten times as much concanavalin A is required to selectively reduce EOGs in the frog (Wood *et al.*, 1983). This may be explained by the presence of a thicker layer of mucus overlying the frog olfactory epithelium than is seen in the rat (Menco, 1980).

#### $\alpha$ -Methyl-D-mannoside protection of EOGs

The results of the mannoside protection experiment seen in figure 6 show that concanavalin A modification of the olfactory response to all three odorants could be prevented by competing for the sugar residue binding site on the concanavalin A molecule with mannoside. This effect is also described elsewhere (Shirley *et al.*, 1987b).

One-way analysis of variance showed for each odour that there was a difference in the  $\langle R \rangle$  values for the three treatments on the Wistar rat (cineole and i-pentanoic acid  $p < 0.001$ , nicotine  $0.01 > p > 0.001$ ). To identify which of the treatments contributed most to this difference, the control  $\langle R \rangle$  values were compared with  $\langle R \rangle$  values for concanavalin A and  $\langle R \rangle$  values for concanavalin A and mannoside, using the Dunnett test for multiple comparisons to a control group (Roscoe, 1975). The results of this test showed that for each odour, only the concanavalin A treatment  $\langle R \rangle$  values were significantly different from the control  $\langle R \rangle$  values (cineole, nicotine and i-pentanoic acid,  $p < 0.01$ ).

Treatment of the epithelium with mannoside after a concanavalin A superfusion does not reverse the concanavalin effect (Shirley *et al.*, 1987b), but the EOGs which were protected in the concanavalin A

and mannoside treatment in this study could be reduced by subsequent treatment with concanavalin A alone (reductions in response of 12%, 27% and 3% for cineole, 38%, 26% and 40% for nicotine and 28%, 42% and 38% for i-pentanoic acid, from three separate preparations). These observations suggest that  $\alpha$ -methyl-D-mannoside is binding to the concanavalin A molecule's sugar residue binding-site, preventing modification of the EOGs.

This is evidence that the olfactory receptors which respond to cineole, nicotine and i-pentanoic acid are glycosylated and/or are close to a portion of sensory membrane which is glycosylated. Other workers have shown that there are glycoproteins unique to sensory cilia and have suggested that these proteins play a role in olfactory reception (Chen & Lancet, 1984).

Nicotine, a key chemical found in the smoke from cigarettes and other tobacco products, has been shown here to act as an odorant in addition to its well-known role as a pharmacological agent. The contribution that nicotine makes to the overall flavour of tobacco smoke and the actual odour quality of pure nicotine has not been investigated. In preliminary experiments on human volunteers, subjects could smell the pure nicotine used in this work (see appendix A).

On the basis of these results, it is reasonable to suggest that other pharmacologically active odorants exist. It is also reasonable to suggest that volatile pharmacological compounds may have interesting effects on the olfactory epithelium and odorant detection which may ultimately be of commercial use.

## Chapter 2a.

### Spatial variation in response to odorants on the rat olfactory epithelium.

#### Introduction

The olfactory epithelium in the rat is located on bony turbinate structures and on the septum which separates the two halves of the nasal cavity. This sensory epithelium is the site of a complex series of events following odorant stimulation, culminating in the generation of an action potential in the primary olfactory neurons. These primary neurons synapse in the olfactory bulb where subsequent processing of the information from the epithelium occurs (Getchell, 1986; Lancet, 1986).

The mechanisms by which the olfactory system can distinguish between the very large number of "smells" found in the environment using a finite number of receptors is still not fully understood. One level at which determination of odour quality can occur is at the initial interaction of odorant with the olfactory epithelium. The layer of mucus which covers the olfactory epithelium will affect odorants which dissolve in it in different ways (Mozell & Jagodowicz, 1973; Getchell *et al.*, 1984) and will therefore affect the rate and concentration at which odorants reach the olfactory receptors. This mode of discrimination between odorants is an example of "imposed" patterning of the stimulus-olfactory epithelium interaction. A

second type of discrimination is more specific, in that the stimulus can be identified through differences in the stimulated receptor populations, in the transduction pathways activated and in the arrangement of neuronal connections to the olfactory bulb.

This specific "patterning" of the response to odorants has been demonstrated in several ways at different levels in the transduction process. Electrophysiological studies have shown that an odorant stimulates more than one receptor type (Shirley *et al.*, 1987a,b) and that the olfactory neurons differ in the range of odorants to which they respond (Revial *et al.*, 1982a,b). *In vitro* experiments using the olfactory odorant-modulated adenylate cyclase also suggest that odorants stimulate a heterogeneous population of receptors and that some odorants are poor stimulants for this particular transduction mechanism (Shirley *et al.*, 1986; Sklar *et al.*, 1986). The electro-olfactogram (EOG), a summated receptor potential from many olfactory neurons, has been used to demonstrate regional differences in response to odorants in the frog (Mustaparta, 1971) and the salamander (Mackay-Sim & Kubie, 1981), and histological studies have shown that the membranous particles of vertebrate olfactory cilia, supposedly the olfactory receptor sites, are unevenly distributed within the epithelium (Menco, 1983). A recent study has demonstrated that specific regions of the olfactory bulb are connected with specific regions within the olfactory epithelium (Pederson *et al.*, 1986). These results support the hypothesis that the olfactory neurons are arranged in a mosaic within the epithelium and that different odorants may stimulate receptors at different regions of the olfactory epithelium. The role of the olfactory

bulb in odorant discrimination should not be overlooked. There is good evidence that coding of the information from the epithelium occurs here also (Kauer & Moulton, 1974; Duchamp, 1982; Duchamp & Sicard, 1984).

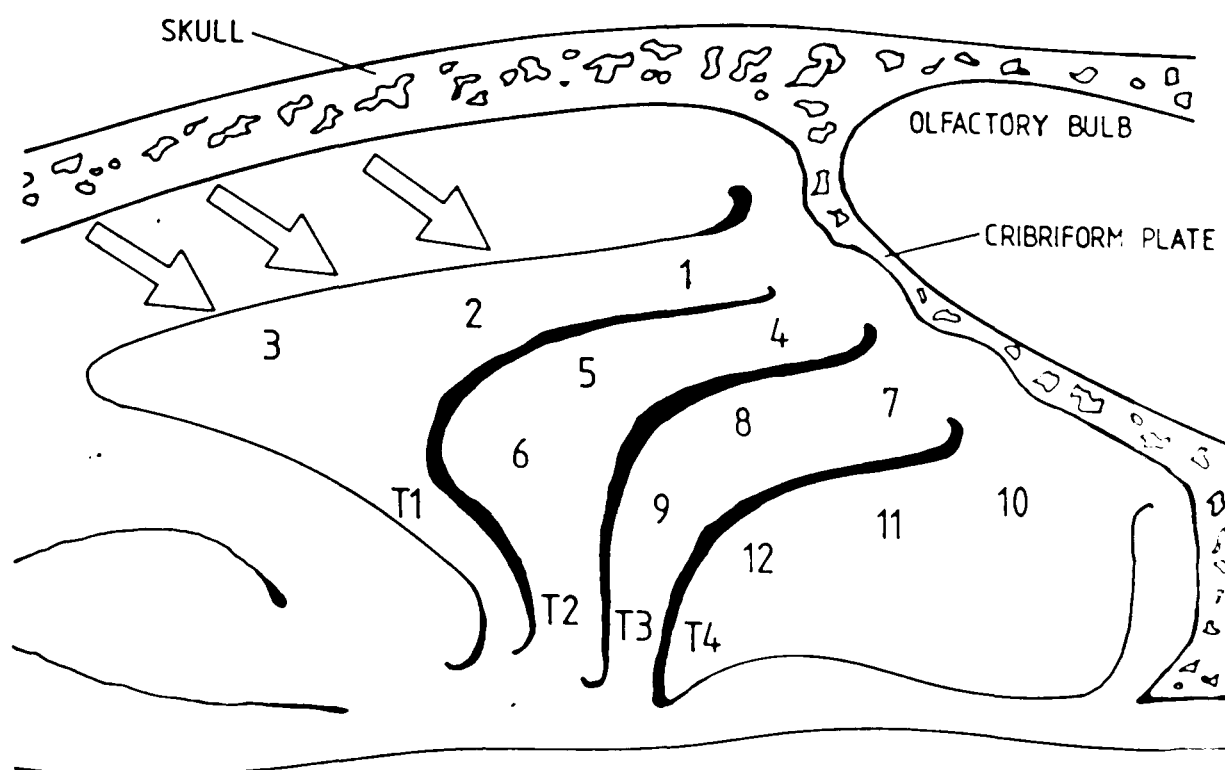
The spatial patterning of the response to odorants by the rat olfactory epithelium is suggested from EOG recordings taken from the olfactory neurons on the dorsal side of the cribriform plate (Thommesen & Doving, 1977). Here I have used the EOG as a measure of the response from twelve positions on the epithelium itself to four odorants with markedly different structures and odours, 1-pentyl acetate, 1-pentanoic acid, cineole and nicotine.

## Materials and Methods

### Recording EOGs from 12 positions on the olfactory turbinates

The *in vitro* preparation, odorants and methods used in this study are as described in chapter 1. The exposed epithelium of each of the four olfactory turbinates was allocated three positions from which it would be possible to record EOGs. These positions were essentially the same in each rat studied, though some variation in topography of the turbinates of different preparations was observed during the study. Thus, there were twelve positions from which EOGs could be recorded (figure 7). The right side of the head was routinely used in this study.

Figure 7. Schematic Diagram of the Olfactory Turbinates Showing 12 Recording Positions



The twelve positions from which EOGs were recorded are marked 1 to 12, three on each of the four turbinate structures, T1-T4. The arrows show the direction from which the odorants were presented to the epithelium.

Initially, EOG recordings were taken from position 8 on every rat studied, after which the electrode was raised and repositioned by movement of the head stage holding the preparation. An identical sequence of the odorants (each presented in duplicate as a one second pulse of vapour followed by a one minute recovery period) was then presented to the new position. By moving the preparation in this manner, the distance between the electrode tip and odorant delivery nozzle was fixed throughout each experiment. The same vapour concentration of each odorant was applied to

each position studied (nicotine 65 nM, cineole 309 nM, i-pentanoic acid 383 nM and i-pentyl acetate 981 nM).

Recordings were made from position 8 in each rat to provide a common reference position for the experiment, since time did not permit me to record EOGs from all twelve positions on each rat studied. The same electrode was used to record from any one preparation but was replaced as required during the study; this will not significantly affect the EOGs recorded. The order of positions from which recordings were taken was as random as possible after the following criteria were obeyed. Neither adjacent positions were used on the same turbinate nor opposite areas on neighbouring turbinates, where the first presentation of odorants may have reduced the sensitivity of the second or subsequent areas to be studied. In a single preparation it was possible to record from four or five positions before there was a risk of the tissue drying out. The olfactory epithelium was not superfused with Ringer solution between recordings from each position.

Discriminant analysis on the results from the study was performed on an IBM 4381 mainframe computer utilising the Statistical Package for the Social Sciences (SPSSx) programmes.

## Results

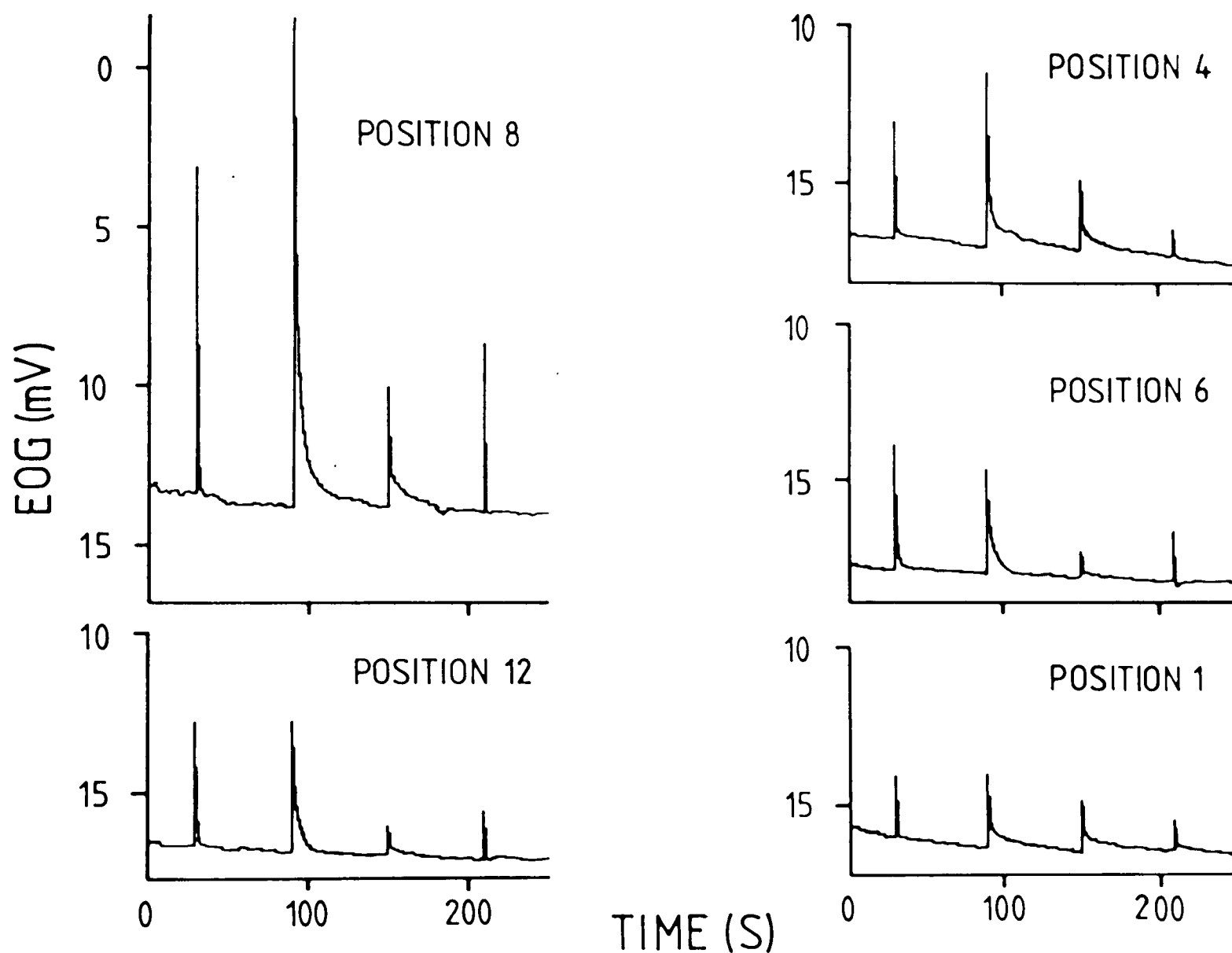
### Variation in EOG with recording position

An example of a typical experiment is shown in figure 8. The EOG traces were recorded consecutively from five positions (positions 8, 12, 4,



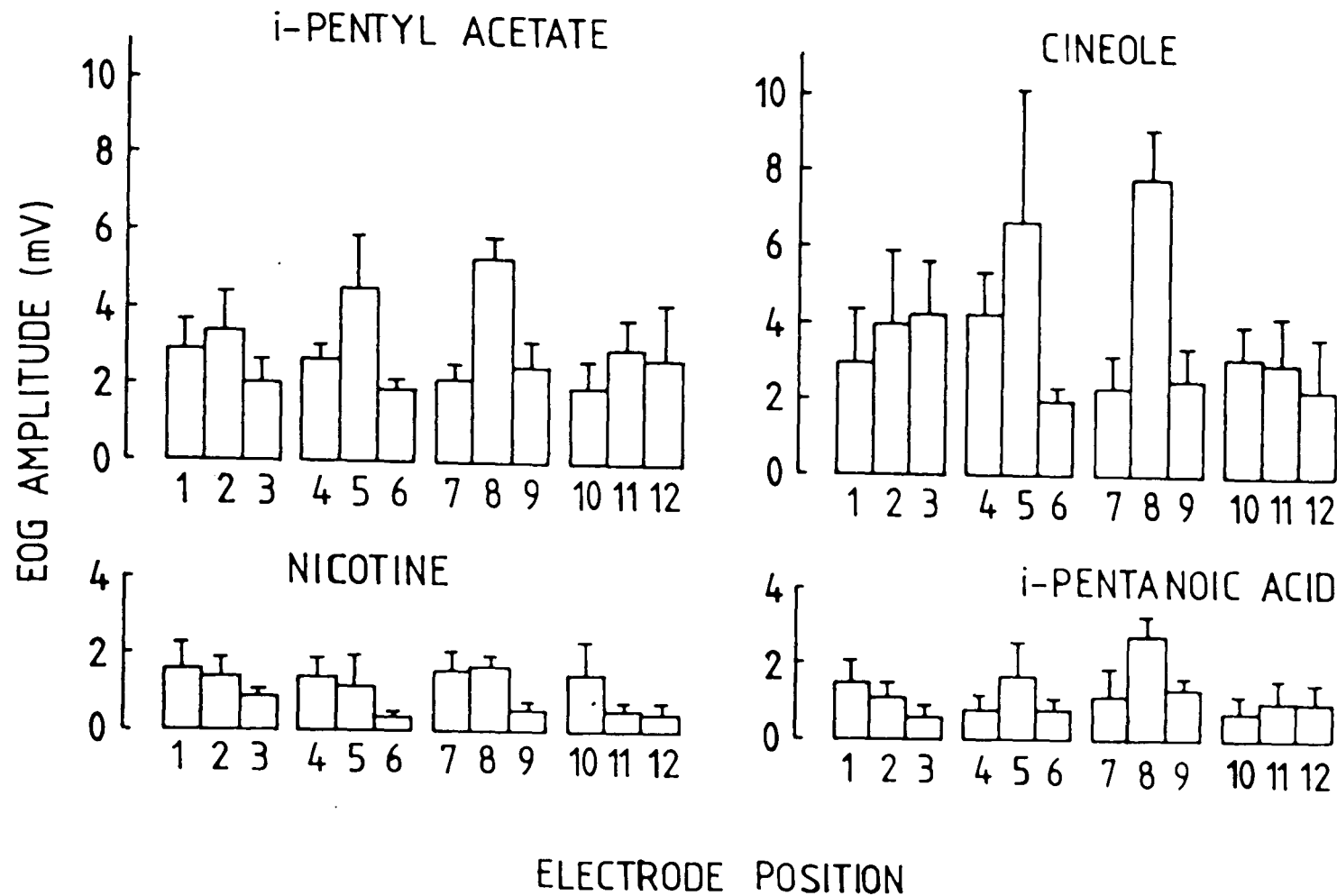
6 and 1 shown in figure 7) on the same rat olfactory preparation and show that there are differences in response of the rat olfactory epithelium to the four odorants at the five positions. In all, nineteen rats were studied.

Figure 8. Spatial Variation in EOG Response



The results shown are from a single Wistar rat half-head preparation. The presentation order (and the vapour phase concentration) of the odorants is from left to right, i-pentyl acetate (981 nM), cineole (309 nM), nicotine (65 nM) and i-pentanoic acid (383 nM). The order of recordings is position 8 first, then 12, 4, 6, and 1.

Figure 9. Mean EOG Versus Recording Position



Values show mean EOG amplitude with the upper half of the 95% confidence interval. The minimum number of presentations to any one position is 10 (nicotine, cineole and i-pentanoic acid to positions 5 and 11) and the maximum is 69 ( i-pentyl acetate to position 8).

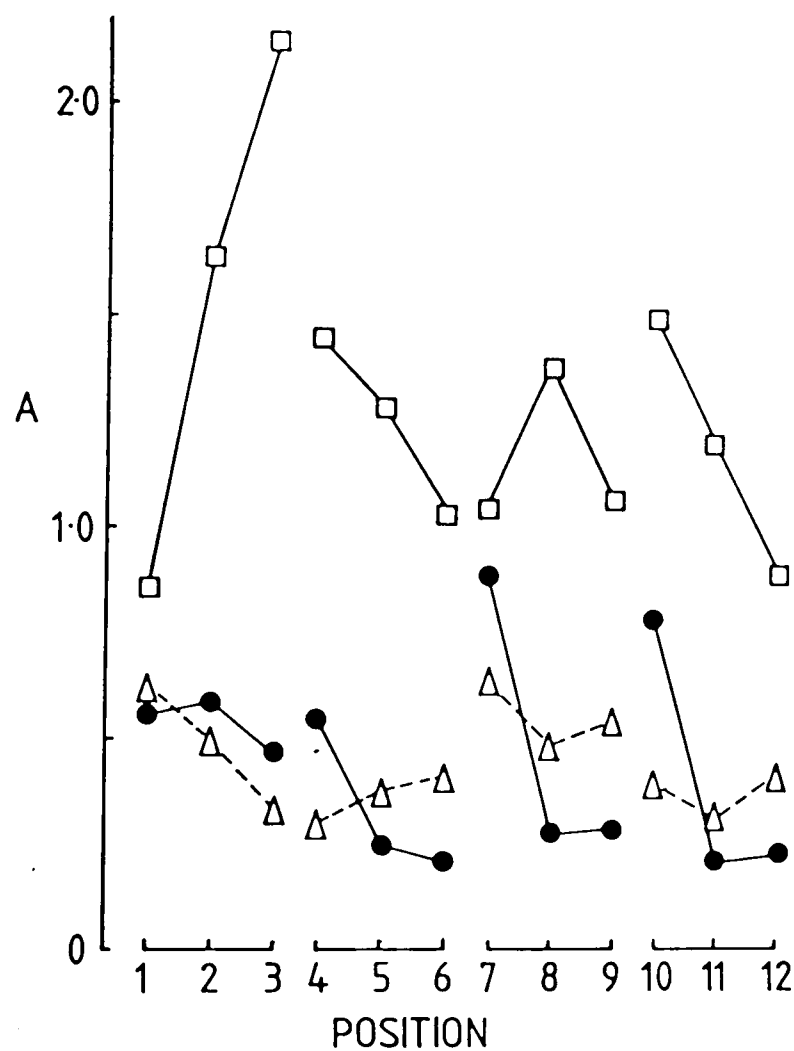
The mean EOG amplitude to the odorants at each position is shown in figure 9. The data was collected from all presentations of odorant made to the olfactory epithelium during the study, typically three presentations of i-pentyl acetate and two each of nicotine, cineole and i-pentanoic acid at each position, remembering that it was not possible to record EOGs from all twelve positions on the same preparation and that position 8 was studied

studied on every preparation. The results shown in figure 9 suggest that there are spatial differences in the amplitude of response to the odorants tested. However, accurate interpretation of these data is difficult when it is realised that in addition to experimental effects (see discussion) the data do not account for variation in response between animals.

In previous studies the  $\langle A \rangle$  value has been used to account for this variation (see chapter 1; Shirley *et al.*, 1987b) and I have applied this analysis here also. The value  $\langle A \rangle$  was calculated for each odorant by normalising the EOG peak amplitude recorded from nicotine, cineole or i-pentanoic acid to the EOG peak amplitude recorded from i-pentyl acetate at the same position. Thus, we are describing the EOG for nicotine, cineole and i-pentanoic acid by a value that is independent of EOG amplitude and is consistent for each odorant between animals.

#### Variation in $\langle A \rangle$ value with recording position

The mean  $\langle A \rangle$  value for each odorant at each position is shown in figure 10 and gives an estimate of changes in  $\langle A \rangle$  value versus recording position. The  $\langle A \rangle$  value can change due to a relative increase or decrease in EOG amplitude measured from one or both of the 'test' odorant (cineole, nicotine or i-pentanoic acid) or 'reference' odorant (i-pentyl acetate). As can be seen from figure 10, the change in  $\langle A \rangle$  value for the 'test' odorants is not identical across the twelve positions, again suggesting that there are spatial differences in response to the test and/or reference odorant on the rat olfactory epithelium.

Figure 10. Mean <A> Value Versus Recording Position

The points shown mean <A> value for □ cineole, ● nicotine and △ i-pentanoic acid. The 95% confidence intervals for each odorant (from positions 1 to 12) are as follows; cineole  $\pm$  0.24, 0.37, 0.54, 0.29, 0.29, 0.20, 0.46, 0.10, 0.18, 0.37, 0.53, 0.23, nicotine  $\pm$  0.28, 0.23, 0.18, 0.20, 0.14, 0.04, 0.49, 0.03, 0.05, 0.41, 0.13, 0.08, i-pentanoic acid  $\pm$  0.42, 0.15, 0.25, 0.10, 0.26, 0.14, 0.41, 0.05, 0.18, 0.19, 0.27, 0.17

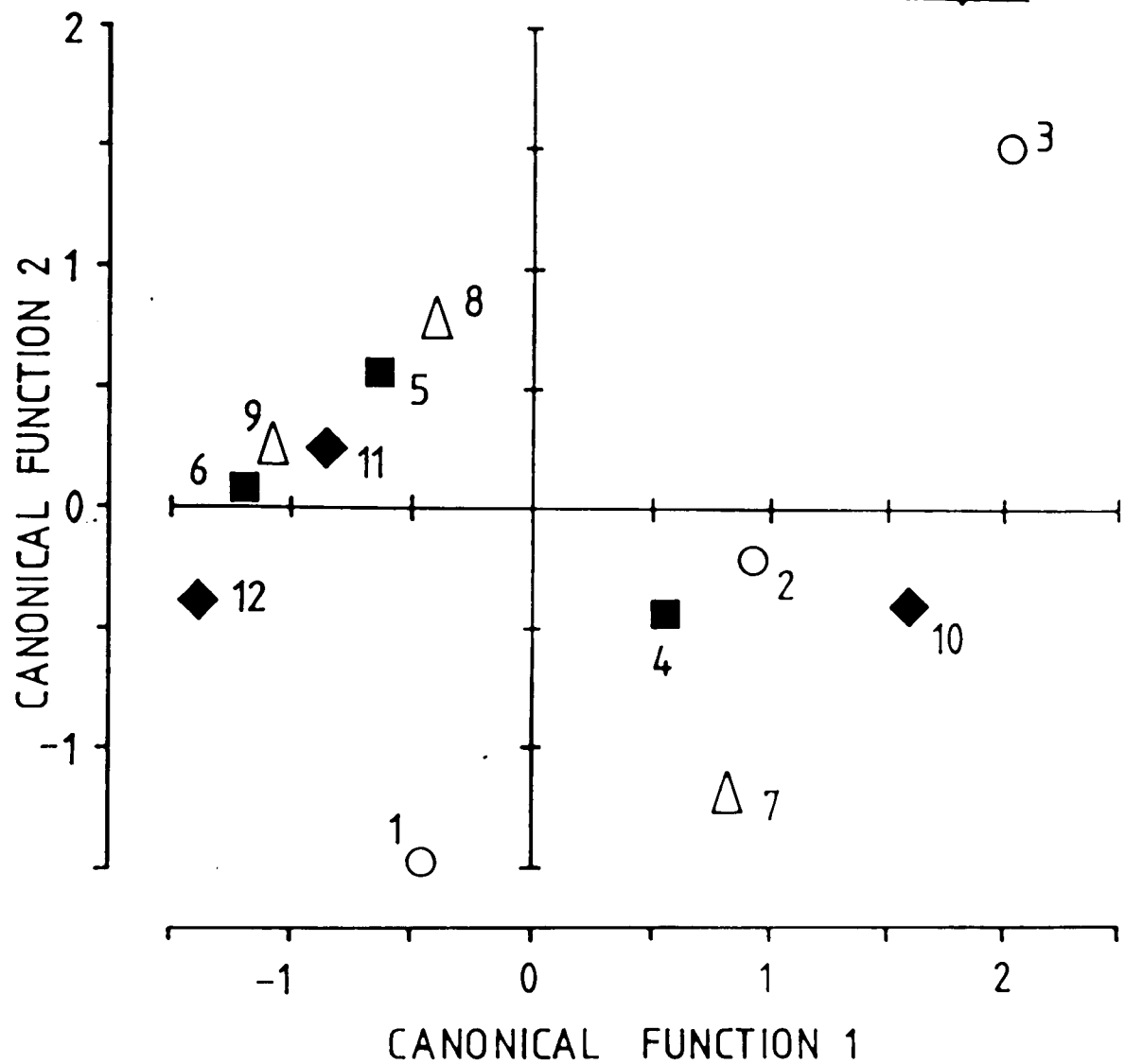
#### Variation in response described by discriminant analysis

The variation in <A> value across the twelve positions for each test odorant may not be identical for each animal tested, thus evidence for spatial patterning may be obscured by simply calculating the mean <A>

value. We therefore analysed the  $\langle A \rangle$  value data from each preparation using discriminant analysis, in which each position studied on each animal was described mathematically by canonical values relating the normalised response ( $\langle A \rangle$  value) of the olfactory epithelium to the three test odorants. The equations used to determine these values calculated the maximum discrimination possible between the  $\langle A \rangle$  values. A plot of the two most discriminating functions on x-y axes represents the movement of the value from the origin during the analysis. The canonical values for each position are then averaged, giving a mean value, the centroid, for each position studied (figure 11). Using this analysis we represent the original EOG data from four odorants in a two-dimensional plot.

Figure 11 shows that the centroid values for the positions do not fall into the same quadrant of the plot, again suggesting that there are regional differences in response to the odorants tested which can be identified by the normalised responses to cineole, nicotine and i-pentanoic acid. The data points used in the discriminant analysis were related to the actual recording position by a number from 1 to 12 (as in figure 1). The analysis classified the data from 33 out of 92 recordings as coming from the expected recording position. The probability that this result has been generated by chance is a function of the poisson distribution and gives  $p \ll 0.001$ . This indicates that there is a similar pattern of response from the nineteen animals used.

Figure 11. Centroid Value Plot Following Discriminant Analysis



Plot of the centroid values for each recording position (mean value for canonical the functions) determined using discriminant analysis of the <A> values for cineole, nicotine and i-pentanoic acid. The shaded symbols represent a negative value for function 3. All points start at the origin before analysis. Numbers 1 to 12 represent the centroid value for the recording position on ○ T1, □ T2, △ T3 and ◇ T4 of figure 7.

Discussion

Spatial variation in response to odorants

The data obtained in this study shows that there is spatial patterning of response to the four odorants tested in both EOG amplitude (figure 9)

and variation in the normalised response (figure 10). These results must be interpreted with caution in order to distinguish imposed patterning (including experimental effects) from the patterning which should be observed if the odorants stimulate different regions of the olfactory epithelium.

The EOGs measured at the twelve positions will be affected by differences in mucus thickness and composition between each recording position, the proportion of non-responsive respiratory epithelium in the regions tested and the position of the recording electrode relative to the earth electrode. Smaller EOGs are likely to be recorded near to the edges of the turbinates due to current leakage being greater in these regions. This should affect the EOG to all four odorants equally. The same is true of the amount of respiratory epithelium at each position (likely to be greatest at positions 3, 6, 9 and 12). The mucus layer covering the epithelium may impose patterning on our preparation in the following way. The response to odorants that have a large water/air partition coefficient (nicotine and 1-pentanoic acid) will be affected to a larger extent by changes in mucus thickness than the less soluble odorants (1-pentyl acetate and cineole). As the mucus thickness increases, the decrease in EOGs to nicotine and to 1-pentanoic acid will be greater than any change in the EOG to cineole and to 1-pentyl acetate. Evidence suggests that in the rat the mucus layer is of uniform thickness (about 5 microns; Menco, 1980). Thus imposed patterning via mucus effects is unlikely to explain large differences in the response measured from the twelve positions, particularly between nicotine and 1-pentanoic acid.

The design of the electrode and stimulus source (Shirley, 1987), although reducing imposed variation in the response, may influence the EOGs in a different manner. Diffusion of the odorants (or even transport; Pevsner et al., 1986) through the mucus may allow adaptation to occur in an adjacent position on the same turbinate. The experimental protocol I used avoided recording from two such positions without leaving time for the odorants to disperse (by recording the EOGs from a position on a different turbinate). If such adaptation had occurred, then the response of an odorant would be reduced at the subsequent recording position. Observations made during the experiment suggested that this was not the case.

#### Use of discriminant analysis to show positional differences in response

The differences between recording position determined by discriminant analysis (figure 11) have no experimental parameter, but are a useful pointer to positions which show large differences in response to others. With reference to the EOG data shown in figure 9, it is possible to discover why these differences have been identified by the discriminant analysis. For example, position 3 is distinguishable from all others because of a relatively larger response to cineole than is expected for a uniform distribution of receptors for all four odorants. Another example is the response to nicotine vapour at position 10 which is larger than expected, whereas the response to i-pentanoic acid vapour at the same position is not, suggesting that imposed patterning in this case is unlikely. In the previous chapter I have shown that the lectin concanavalin A reduces the EOG to nicotine, cineole and i-pentanoic acid to



different extents, suggesting that the three odorants stimulate different combinations of olfactory receptor. Thus, the unrelated variation in response over the twelve positions to each of the three odorants seen here is not unexpected.

The relative arrangement of the centroid values for each turbinate is also interesting. The first turbinate (positions 1,2 and 3) is easily distinguished from the others on the basis of the response to the odorants tested. The other three turbinates are not so easily separated by canonical functions 1 and 2, though all have differences in response between the anterior and posterior of each turbinate. Such positional differences have been observed previously in the salamander (Mackay-Sim & Kubie, 1981). Values for canonical function 3 for each centroid do appear to separate the responses of turbinates 1 and 3 from those of turbinates 2 and 4. Such patterns may be expected from a mosaic of receptors within the olfactory epithelium.

These observations suggest that specific patterning of response to the odorants is seen on rat olfactory epithelium. This patterning is most likely explained by differences in receptor populations between the positions studied and is consistent with other studies suggesting this type of arrangement of olfactory receptors in the rat and other species (Mackay-Sim & Kubie, 1981; Pedersen *et al.*, 1986; Thommesen & Doving, 1977).

In addition, the result for nicotine supports the conclusion in chapter 1 that nicotine stimulates olfactory receptors of rat olfactory epithelium in a manner similar to other odorants.

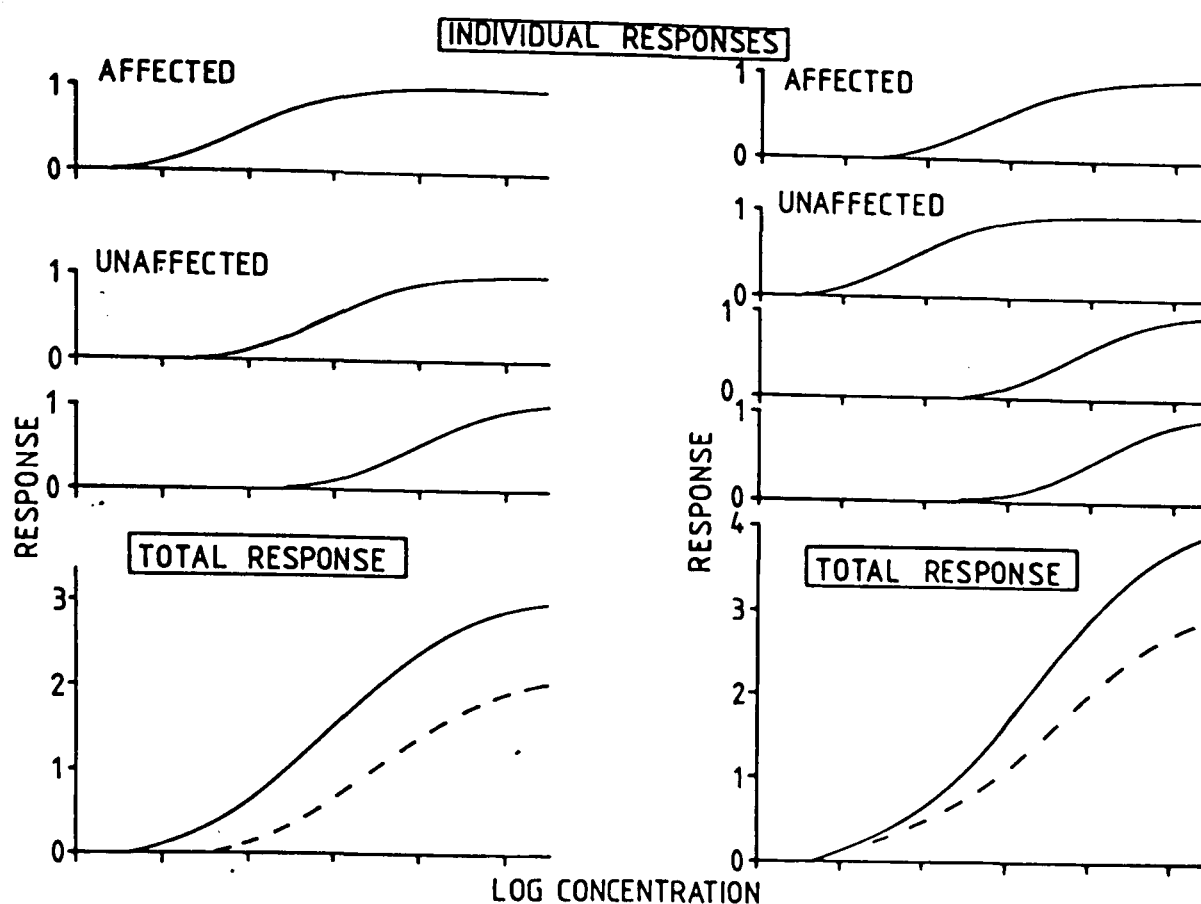
## Chapter 2b.

The effect of concanavalin A on the rat electro-olfactogram at varying odorant concentrations.

### Introduction

Any study of a receptor system is incomplete without an estimate of the receptor's affinity for a given ligand. This parameter is difficult to determine for olfactory receptors for several reasons. First, there are many possible ligands which stimulate an unknown number of receptors with different affinities for each ligand, and secondly, the concentration of the ligand at the receptor is difficult to determine accurately. These points have already been discussed in the General Introduction and in this section of the thesis.

The EOG is a summated receptor potential, i.e. the amplitude of the response measured is related to the interaction of an odorant with several receptor types on many sensory neurons (see Ottoson, 1970; Shirley *et al.*, 1987b). Each receptor type stimulated by a given odorant may respond to that odorant over a particular range of odorant concentrations. This idea is illustrated in figure 12.

Figure 12. Examples of Multiple Receptor Response

Two examples are shown, in each case the lower curve shows the total response of the stimulated receptors. The broken curve shows the total response when one of the receptor types is affected by a reagent and its contribution to the total response is lost. The difference between the total response before treatment and after treatment with the reagent represents the response profile of the affected receptor type.

Clearly it is difficult to assign any portion of a concentration-response relationship curve to a single receptor type, except at high odorant concentrations where a single receptor type is thought to be involved (Senf *et al.*, 1980). These authors investigated the shape of the EOG amplitude versus odorant concentration curve for a series of alcohols, and found that at high odorant concentrations a single dissociation

constant (ranging from 1 to  $10^{-5}$ ) best explained the concentration-response relationship. Their results also suggested that odorant interaction with an olfactory receptor was mainly through hydrophobic effects.

The lectin concanavalin A has been shown to reduce the EOG to many odorants to different extents (Shirley *et al.*, 1987b). These results may be explained by the concanavalin A interacting with one or more receptor type which responds to a given odorant. It is possible that treatment of the olfactory mucosa with the lectin may change the shape of the EOG amplitude versus concentration curve in a manner related to the loss of the lectin sensitive receptors. This possibility was investigated on the rat half head olfactory preparation described earlier, using nicotine, n-butyl cyanide, i-pentanoic acid, methyl disulphide, i-butyl mercaptan, i-butyraldehyde, hexan-1-ol and i-pentyl acetate as the odorants. The results for methyl disulphide, i-butyl mercaptan and i-butyraldehyde were obtained by Dr. S.G. Shirley and the result for hexan-1-ol by Mr. M.A. Wood.

## Materials and Methods

### Chemicals

The nicotine used was as described in chapter 1. n-Butyl cyanide (98%) and i-pentanoic acid (98%) were from Fluka, methyl disulphide (99%), i-butyl mercaptan (97%), i-butyraldehyde (99%), hexan-1-ol (98%) and i-pentyl acetate (97%) were all obtained from Aldrich.

**Precautions when using low concentrations of odorant**

The protocol used was as described earlier except for the following modifications and precautions. Since low concentrations of odorants were being used, the olfactometer was dis-assembled, the components washed in chloroform and baked *in vacuo* at 120°C to ensure as little contamination by residual odorants as possible. The response of the half head preparation itself was used to determine the cleanliness of the apparatus before proceeding with the next odorant. The apparatus was accepted as clean when the "clean" air used in the experiments (see chapter 1 for details on the cleaning procedure) gave an EOG response less than the EOG response to the lowest concentration of odorant used. The odorant was presented to the epithelium as a one second pulse of vapour followed by a one minute recovery period, and was applied in order of increasing concentration with the standard odorant, i-pentyl acetate, presented at regular intervals within this sequence. After the initial sequence of odorants had been applied, the tissue was treated with concanavalin A or Ringer solution only as described earlier.

The  $\langle A \rangle$  value for each duplicate presentation of odorant was calculated both before and after concanavalin A treatment. The difference between these values was termed delta  $\langle A \rangle$  and represented the effect of the concanavalin A on the portion of the EOG determined by the lectin sensitive receptor(s). Estimation of the mucus concentration of the odorants is described earlier (see page 45).

## Results

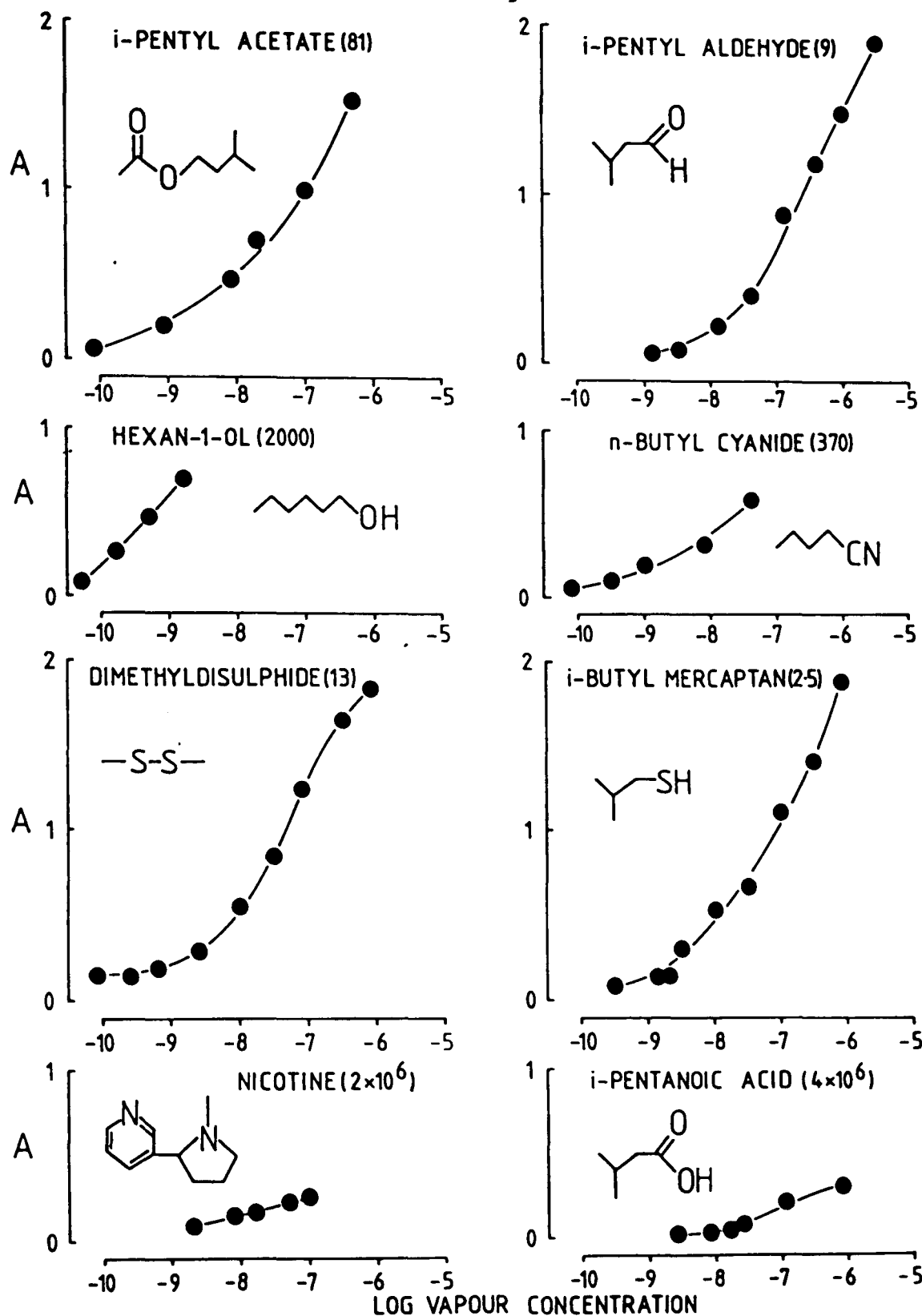
### Change in $\langle A \rangle$ value with increasing odorant concentration

The normalised response ( $\langle A \rangle$  value) versus concentration relationship for each odorant is shown in figure 13 and is consistent with the result expected for a multiple receptor response over a wide concentration range. These data can be fitted to the equation  $\log \langle A \rangle = m \times \log [\text{odorant}] + \text{constant}$ , and used to estimate the vapour concentration of odorant required to give an EOG equal in amplitude with the EOG from the standard odorant (i-pentyl acetate), SV, and the corresponding mucus concentration, SM (calculated by Dr. S.G. Shirley). This data is shown in table 4.

Table 4. Concentration of odorant required to give an EOG equal in size with the EOG from the standard odorant.

$r$  = correlation coefficient and  $m$  = slope

Odorant	$r$	$m$	$-\log SV$	$-\log SM$
methyl disulphide	0.988	0.32	7.1	6.0
i-pentyl aldehyde	0.969	0.47	6.4	5.4
i-butyl mercaptan	0.980	0.42	7.0	6.6
i-pentyl acetate	0.989	0.38	7.1	5.2
hexan-1-ol	0.968	0.49	8.5	5.2
i-pentanoic acid	0.956	0.46	5.1	0.3
n-butyl cyanide	0.997	0.37	6.8	4.3
nicotine	0.984	0.29	5.1	0.4

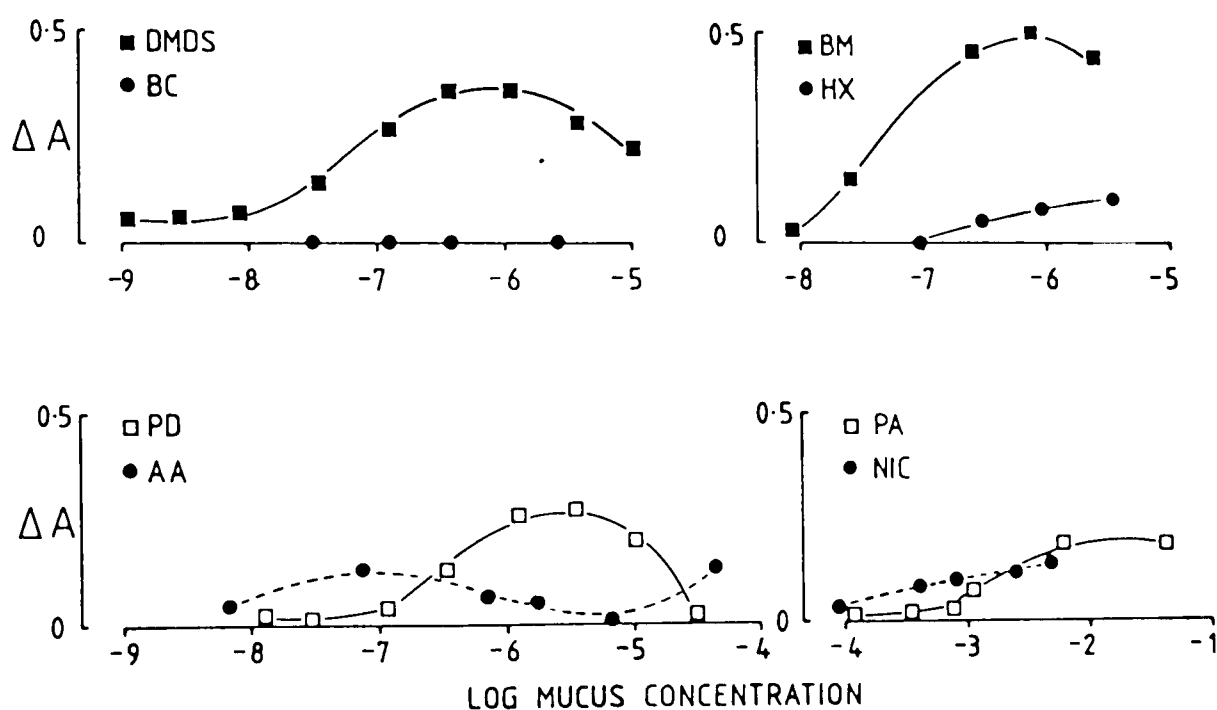
Figure 13. <A> Value Versus Odorant Vapour Concentration

Each point represents the mean  $\langle A \rangle$  value from at least five animals. The figure in parentheses is the water / air partition coefficient. 95% Confidence intervals are not shown for clarity. Typical values (for low to high concentrations of dimethyl disulphide) are  $\pm 0.04, 0.04, 0.05, 0.08, 0.15, 0.19, 0.21, 0.24, 0.25$ .

### Concanavalin A induced change in $\langle A \rangle$ value ( $\Delta \langle A \rangle$ )

The EOG response of n-butyl cyanide was unaffected by the concanavalin A treatment, but for the other odorants the lectin caused a reduction in EOG amplitude, particularly at the higher odorant concentrations tested. The mean concanavalin A induced change in response ( $\Delta \langle A \rangle$ ) versus estimated mucus concentration of odorant is shown in figure 14.

Figure 14.  $\Delta \langle A \rangle$  Versus Mucus Concentration of Odorant



Mucus concentrations were determined by multiplying the vapour concentration by the water / air partition coefficient, except for nicotine and i-pentanoic acid where a factor of 50,000 was used (see Materials and Methods of Chapter 1 for an explanation). Points show mean  $\Delta \langle A \rangle$  value calculated in part from the data in figure 13.

DMDS - dimethyl disulphide; BC - n-butyl cyanide; BM - i-butyl mercaptan; HX - hexan-1-ol; PD - i-pentyl aldehyde; AA - i-pentyl acetate; PA - i-pentanoic acid; NIC - nicotine



The maximum concanavalin A induced change in EOG occurred at approximately 1  $\mu$ M for i-butyl mercaptan, methyl disulphide and i-pentyl aldehyde and probably for hexan-1-ol although the data points for this odorant were not continued to a high enough concentration to confirm this.

The results in figure 14 show that the EOG to the standard or reference odorant i-pentyl acetate over a range of concentrations is affected by the lectin. This reduction in EOG to the standard odorant was small and so will not obscure large effects the lectin has on the response to the other odorants. The  $\langle L \rangle$  value (see Chapter 1 for a definition) for the experiments of  $0.80 \pm 0.20$  (mean  $\pm$  standard deviation,  $n=45$ ) also suggested some non-specific and receptor specific effects of the lectin on the response to the standard odorant. The control washes with Ringer solution only had no significant effect on the EOGs to any of the odorants at any concentration of odorant.

Concanavalin A induced changes in the EOGs to nicotine and i-pentanoic acid occurred at higher estimates of the mucus concentration of the odorants than for the other affected odorants. These hydrophilic odorants also required a higher vapour concentration to elicit a response from the *in vitro* preparation.

## Discussion

It must be remembered that these experiments were performed at the limits of the technique and consequently, the results are subject to some errors. However, the experiments used a novel approach in investigating odorant-binding affinities of olfactory receptors and the results are worth consideration.

### Use of delta $\langle A \rangle$ to estimate olfactory receptor affinities

The results of this study clearly show that a portion of the EOG response to the odorants at varying concentrations can be attributed to one or more olfactory receptor which is sensitive to concanavalin A treatment. For the odorants whose concentrations in the mucus covering the epithelium can be estimated (the odorants with a mucus/air partition coefficient much less than 50,000), the delta  $\langle A \rangle$  values versus concentration curve can be taken to represent the binding affinity of the receptor(s) for those odorants. For the odorants whose mucus concentrations are more difficult to estimate, the delta  $\langle A \rangle$  versus concentration curve will give a higher estimate of the receptor's affinity for the odorants than is actually the case.

The data points for some of the odorants were not measured at high enough concentrations to analyse the receptor affinity (nicotine and hexan-1-ol). For methyl disulphide, 1-butyl mercaptan, 1-pentanoic acid and 1-pentyl aldehyde the delta  $\langle A \rangle$  values can be fitted to a curve which is best described by the response of a single concanavalin A sensitive

receptor. These curves (calculated by Dr. S. Shirley using non-linear regression analysis), fitted to the equation for an ideal receptor;

$$\Delta \langle A \rangle = m \times (c/c + K_D)$$

where  $m$  is a constant and  $c$  is the odorant concentration, (curves not illustrated) show that the dissociation constants for the concanavalin A sensitive receptors for 1-butyl mercaptan, methyl disulphide and 1-pentyl aldehyde are in the order of 100 nM.

The dissociation constant for the 1-pentanoic acid concanavalin A sensitive receptors appears to be greater, but may be overestimated (see above). This will also be the case for nicotine. It is possible that these more soluble odorants are poor stimulants for the olfactory receptors because hydrophobic effects are involved in the interaction of an odorant with the binding site.

The results of this study can be explained in two ways, either that there are several types of concanavalin A sensitive receptor with similar affinities for different odorants or that there is a single class of concanavalin A sensitive receptor which responds to many odorants. The latter possibility is more likely, though it is difficult to substantiate this claim on the basis of these results. A single receptor type would not necessarily be stimulated to the same extent by different odorants at the same concentration (see the differences in the amplitude of  $\Delta \langle A \rangle$  at saturation for the insoluble odorants shown in figure 14 and also the data shown in table 4).

**Effect of change in pH on the nicotine EOG**

To investigate the possibility that a change in pH may affect the EOG to nicotine (in particular that at higher pHs the EOG may be larger than at lower pHs), a further experiment was done. The olfactory mucosa was rinsed with Ringer solution buffered to extremes of pH (pH 6.4 using citrate or to pH 9.2 using serine), the liquid removed by aspiration and the vapour of nicotine, 1-pentyl acetate, 1-pentanoic acid or cineole (vapour concentrations as shown in Chapter 1) was then presented to the olfactory mucosa. The supposed changes in mucus pH did not cause selective, pH-dependent changes in  $\langle A \rangle$  value for nicotine or any of the other odorants (four experiments). The actual pH of the mucus following superfusion was not measured. Thus, the effect on the EOG of varying the amount of nicotine free base in the mucus remains unclear.

## Section 2.

## Chapter 3.

Ligand binding and the study of receptors.  
Use of the filtration binding assay to study  
the binding of  $^3\text{H}(-)$ nicotine to olfactory  
membranes.

### Introduction

#### Ligand binding studies

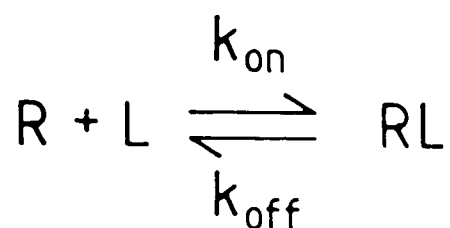
Ligand binding studies are a powerful experimental technique which can be used to study properties of receptors (for a theoretical approach to receptor studies see Boeynaems & Dumont, 1980). Receptors are "specific cellular components that interact with specific ligands" (Levitzki, 1984) and are found both associated with membranes and free within the cytoplasm. Most receptors are thought to be proteins and are often glycosylated. Membrane-associated receptors are influenced by the lipids of the membrane, particularly when the response following stimulation

involves interaction of several membrane components. Receptors are a vital component in the biochemical communication within and between cells, being the link between a specific stimulating ligand (agonist) and an effector pathway causing a specific biochemical response. There are similarities between receptor binding sites and allosteric sites on enzymes, where the binding of a ligand modulates the activity of the enzyme (Levitzki, 1984).

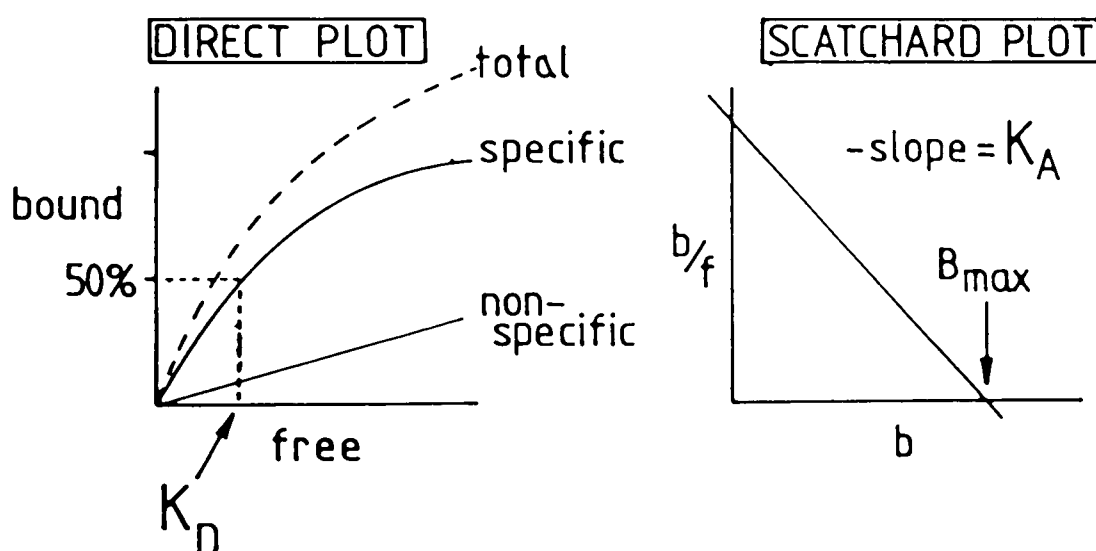
Typical ligands include neurotransmitters (e.g. acetylcholine, dopamine, noradrenaline), hormones (e.g. insulin, prostaglandin) and growth factors (e.g. nerve growth factor). By labelling the natural ligand with isotopes (e.g.  $^{14}\text{C}$ ,  $^3\text{H}$ ,  $^{32}\text{P}$ ,  $^{125}\text{I}$ ) the amount, location and characteristics of a receptor can be studied. In many cases however, the natural ligand is not easily labelled, is unstable or cannot be isolated in sufficient quantity for use in the test-tube. In this case the biochemist has looked for natural or synthetic compounds which also bind to the same receptor but which can be used more easily in the laboratory. The identification of ligands which bind to the receptor but do not stimulate the effector mechanisms (competitive antagonists in particular) has made it easier to measure the appropriate receptor for two reasons, (1) the antagonist binds to the receptor at lower concentrations than the agonist and (2) the antagonist binding to the receptor involves tighter binding than binding of the agonist. In many cases agonists and antagonists can be used to distinguish between different classes of a receptor (e.g. muscarinic

acetylcholine receptors, Birdsall et al., 1983; opiate receptors, Barnard & DeMouliou-Mason, 1983).

There are many different types of ligand binding study which may be used in these experiments, including "filtration", "centrifugation", "equilibrium dialysis" and "molecular filtration" (Levitzki, 1984; Cattabeni & Nicosia, 1984). The most commonly used techniques from these choices are the filtration binding assay and the centrifugation binding assay. Both techniques separate ligand bound to the receptor (RL) from ligand free in solution (L), thereby giving a measure of the amount of ligand associated with receptor in a given tissue preparation. This information can then be used to calculate various parameters which describe the binding of the particular ligand (e.g. the dissociation constant,  $K_D$ , the amount of receptor in a preparation,  $B_{max}$ , the association and dissociation rate constants,  $k_{on}$  and  $k_{off}$ , and the half-life of the RL complex,  $t_{1/2}$ . See figure 15). The filtration assay is most convenient, with the ease and speed of separation enabling the experimenter to do many replicates in any one period of time. The centrifugation assay on the other hand is not as convenient since few replicates can be done in the same period of time and the level of background (non-specific) binding of the ligand to other binding sites in the tissue or apparatus is sometimes high. Non-specific binding is usually measured by including an excess of unlabelled agonist or antagonist in the assay. Both the filtration and centrifugation assays can be used to measure ligand binding to soluble and membrane-associated receptors by minor adjustments in the experimental procedure (e.g. see Bruns et al., 1983; Levitzki, 1984).

Figure 15. Calculation of Binding Parameters from Experimental Data

$$K_D = \frac{1}{K_A} = \frac{[R][L]}{[RL]} \quad \text{at equilibrium}$$



$$K_D = \frac{k_{\text{off}}}{k_{\text{on}}} \qquad t_{1/2} = \frac{\ln 2}{k_{\text{off}}}$$

$$k_{\text{on}} \text{ in } \text{sec}^{-1} \cdot \text{M}^{-1} \text{ and } k_{\text{off}} \text{ in } \text{sec}^{-1}$$

Another technique which can be used to study ligand-receptor interaction is autoradiography on slices of tissue incubated with the labelled ligand. Although this technique is more complex and requires more expensive equipment and computer software than is needed for the equivalent



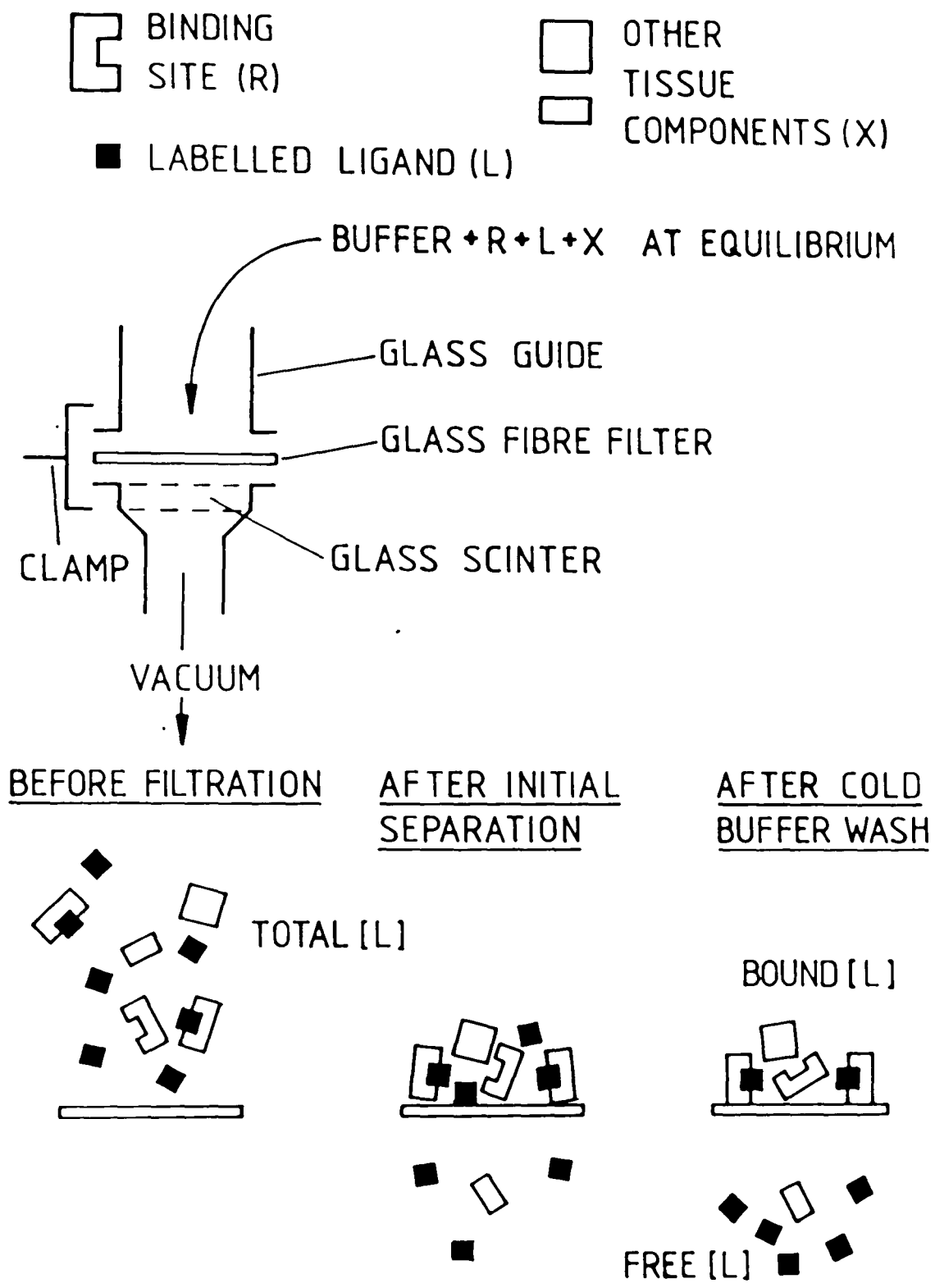
assay in the test tube, it gives excellent results ranging from binding plots to localisation of the receptor sites to specific regions in a tissue. For example, this technique has been used to study the binding of  $^3\text{H}(-)$ nicotine (Clarke *et al.*, 1984) and of  $^3\text{H}$ -thyrotropin-releasing hormone (Sharif & Burt, 1985) to ultra thin slices of rat brain.

Other workers have studied the binding of ligands to olfactory tissue preparations using ligand binding techniques such as molecular filtration on Sephadex gel columns (e.g. Persaud *et al.*, 1981; Pelosi *et al.*, 1982; Wood & Dodd, 1984), centrifugation (Fesenko *et al.*, 1985) and filtration through glass fibre filters (e.g. Hedlund & Shepherd, 1983; Anholt *et al.*, 1984; Pevsner *et al.*, 1985).

#### Principles of the filtration binding assay

In the filtration binding assay the solution containing the receptor preparation and labelled ligand is filtered rapidly through a filter under vacuum (figure 16). This separates the bulk of unbound (free) ligand from ligand bound to the receptor (which is retained on the filter), though it is usual to then wash the filter with a large volume of buffer to ensure maximum separation. To reduce dissociation of the ligand from the receptor trapped on the filter the wash buffer is usually used ice cold. Untreated filters (typically glass fibre) can be used to study membrane-associated receptors, but must be pre-treated with polyethyleneimine or polylysine to enable soluble receptors to stick to the filter (Bruns *et al.*, 1983; Levitzki, 1984).

Figure 16. Filtration Assay Apparatus and it's Use



If the ligand is bound too loosely to the receptor, it may dissociate from the receptor during the separation and wash procedure. A crucial factor in deciding whether the filtration assay can be used to study a particular ligand-receptor interaction is the dissociation rate constant for

the binding,  $k_{off}$  from which the half-life ( $t_{1/2}$ ) of the ligand receptor complex can be determined (figure 15). A lower limit for  $t_{1/2}$  of 15 seconds has been proposed, given that the association rate constant is the diffusion controlled value of  $1-5 \times 10^8 \text{ sec}^{-1} \text{ M}^{-1}$  (Levitzki, 1984). The  $K_D$  of the binding in this situation would be in the order of nanomolar. If the association rate constant is smaller than this, then the lifetime of the receptor-ligand complex will be greater and the filtration assay may be a suitable method for separating the "bound" ligand from the "free" ligand.

In this chapter, determination of the best experimental conditions to study  $^3\text{H}(-)$ nicotine binding to olfactory membrane preparations is described in detail. Initially, the binding of the muscarinic acetylcholine receptor antagonist quinuclidinyl benzilate (QNB) to brain and heart membranes was studied to compare my results using the filtration assay with published results. The binding of this muscarinic receptor antagonist to olfactory membranes described by Hedlund & Shepherd (1983) was also investigated.

## Muscarinic Acetylcholine Receptor

### Materials & Methods

[3- $^3\text{H}$ ]( $\pm$ )Quinuclidinyl-[phenyl-4- $^3\text{H}$ ]-benzilate, specific activity 32 Ci/mmol, was obtained from Amersham International, U.K. and atropine sulphate and oxotremorine were from Sigma, U.K. All other chemicals used were of analytical grade.

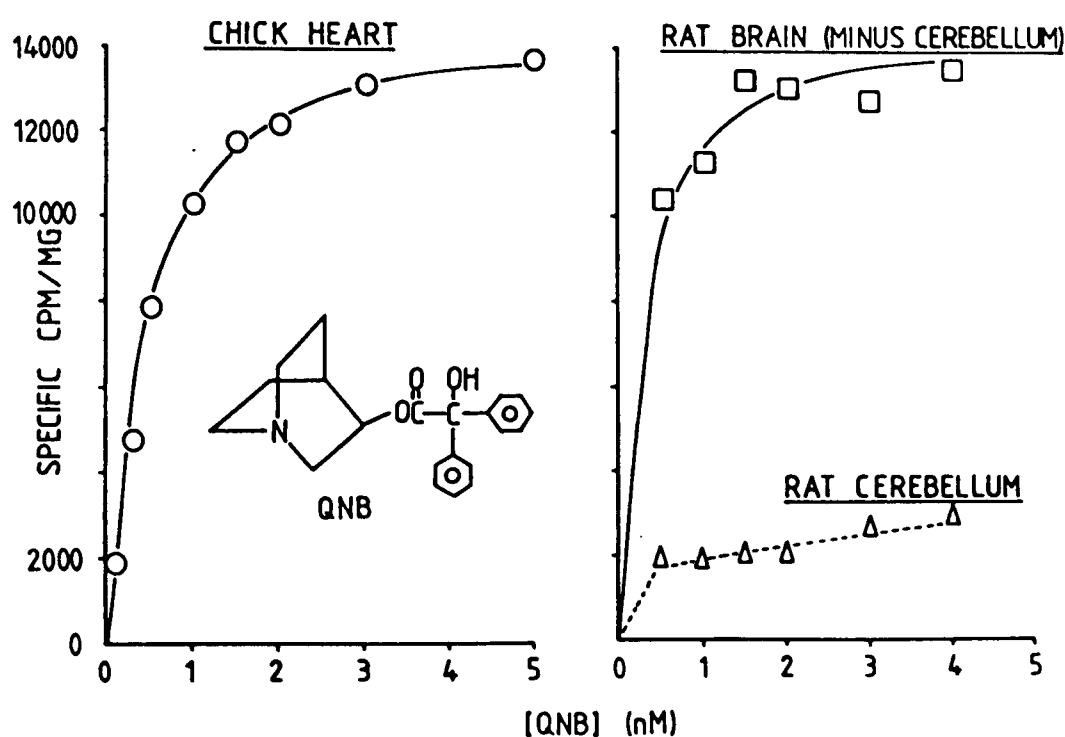
Male Wistar rats, 200-250g, were from Harlan-Olac, U.K. The species of chick used was not known but was a standard laboratory strain.

The binding assay conditions and preparation of membranes was as described elsewhere for rat brain (Yamamura & Snyder, 1974) and chick heart (Galper *et al.*, 1977).

### Results & Discussion

Binding plots from single experiments to investigate the binding of  $^3\text{H}$ -QNB to chick heart membranes, rat brain (minus cerebellum) and rat cerebellum are shown in figure 17. These preliminary results were comparable with the results obtained by other workers and showed that the filtration binding assay apparatus in this laboratory could be used to study ligand binding to receptors.

Figure 17. Binding of  $^3\text{H}$ ( $\pm$ )QNB to Chick Heart and to Rat Brain



The muscarinic acetylcholine receptors that have been identified in the olfactory mucosa of the salamander are found at low levels (80 fmol/mg homogenate protein, Hedlund & Shepherd, 1983) and are thought to play a

role in mucus secretion (Getchell, 1986). The small amount of receptor and of olfactory material prepared from the rat made binding studies with  $^3\text{H}$ -QNB impractical and consequently these experiments were not continued. The preliminary results (not shown) were inconclusive but suggested that a binding site for  $^3\text{H}$ -QNB may be present at low levels in the olfactory mucosa of the rat.

### $^3\text{H}(-)\text{Nicotine}$ Binding to Olfactory Membranes - some experimental considerations

#### $^3\text{H}(-)\text{Nicotine}$

$(-)\text{-}[N\text{-methyl-}^3\text{H}]\text{Nicotine}$  , specific activity 78.4 Ci/mmol, was obtained from Amersham International in 250  $\mu\text{Ci}$  amounts. On receipt, the  $^3\text{H}(-)\text{nicotine}$  was aliquoted into 10 or 20  $\mu\text{Ci}$  amounts in a polypropylene tube (Sarstedt), sealed under nitrogen and stored at  $-20^\circ\text{C}$ . On the day of use the solvent (ethanol) was removed under a gentle stream of nitrogen (caution: nicotine evaporates, see chapter 1) and the  $^3\text{H}(-)\text{nicotine}$  dissolved in the buffer for the assay. Only freshly made solutions of  $^3\text{H}(-)\text{nicotine}$  were used for all major experiments. The radiochemical purity of the  $^3\text{H}(-)\text{nicotine}$  on supply from Amersham was 95.4% and 93.5% in successive purchases of the same batch.

#### $(-)\text{Nicotine}$

$S(-)\text{Nicotine}$  was purchased from Sigma as a brown liquid (quoted as >98% pure) and redistilled before use to a clear liquid (>99% pure as

determined by capillary gas-chromatography; for full details see appendix A). This (-)nicotine was stored in 1-2 ml amounts in clean glass vials, sealed under nitrogen, protected from the light by aluminum foil and kept at -20°C. Discoloured samples of nicotine were not used in the experiments.

#### Preparation of buffers

All chemicals used were of the highest quality available. Glassware and other re-usable apparatus were washed overnight in 5% Decon-90, rinsed three times in distilled water and once in de-ionised distilled water (single distilled water passed through a charcoal column, two ion-exchange columns, an organic "scavenger" column and finally through a millipore filter; final conductivity 18 M.Ohms.cm<sup>-1</sup>). This "pure" water was also used to make all buffer solutions and only freshly made buffer was used in the experiments.

#### Protein assay

Protein was assayed by the method described by Hartree (1972) using bovine serum albumin as standard. The chemistry of the Folin reaction has been discussed elsewhere (Legler *et al.*, 1985). A standard line determined for 10 to 70 µg of bovine serum albumin was measured each time the protein assay was performed (20µl of the olfactory 1000×g supernatant typically contained protein within this range). The buffer of the preparation had no effect on the assay.

### Olfactory membrane preparation

The procedure to prepare olfactory membranes was based around minimal sonication of the dissected and rinsed olfactory turbinates, the underlying principle being to disrupt the surface layer of cells of the olfactory epithelium and to remove the olfactory cilia (see figure 1). The effect of sonication on the olfactory cilia has not been fully characterised in this laboratory, though this procedure has been used to prepare membranes which contain an odorant-modulated adenylate cyclase activity (Shirley *et al.*, 1986; Shirley *et al.*, 1987c,d).

The final procedure is detailed in chapter 4, but is essentially as follows. The ethmoturbinates were removed from a freshly killed Wistar rat and placed in 10 volumes of ice cold phosphate-saline buffer containing 1 mM EGTA, the pH of which is discussed later. The EGTA was included to reduce blood clotting and since it was thought to reduce the amount of mucus covering the olfactory epithelium (observations in this laboratory). After gentle washing in two changes of this buffer and one in buffer minus the EGTA, the turbinates were sonicated. An MSE 100W disintegrator was used with an exponentially tapered probe (3mm tip diameter), giving a meter reading of 12 microns peak-to-peak when used on the medium power setting. This whole procedure was done in the same polypropylene tube. Following sonication the liquid was decanted and the turbinates rinsed with buffer minus EGTA. The pooled liquid was then centrifuged at 1000×g for 15 minutes at 4°C and the supernatant used in the studies without further treatment. This preparation was kept on ice until used in the experiments.

Similar results for the binding of  $^3\text{H}(-)\text{nicotine}$  were observed for supernatant which was tested immediately following the centrifugation step and from the same supernatant tested after storage on ice for up to two hours.

#### $^3\text{H}(-)\text{Nicotine}$ binding assay

The conditions for the *preliminary* assays were as follows. Early experiments had shown that very little  $^3\text{H}(-)\text{nicotine}$  binding to the olfactory 1000 $\times$ g preparation could be measured at low (0.1-10 nM) concentrations of ligand and at pH 7.5 (total cpm measured per incubation with a limited amount of protein). Therefore, the full specific activity  $^3\text{H}(-)\text{nicotine}$  was "diluted" with unlabelled  $(-)\text{nicotine}$  to enable binding at higher concentrations of nicotine to be studied. In the preliminary assays, nicotine at a final concentration of 97nM at 10 Ci/mmol was used, with non-specific binding determined in the presence of 1 mM unlabelled nicotine (similar data was obtained using 0.1 mM nicotine). The assays were conducted in tubes containing buffer, membranes and nicotine to a final volume of 250 $\mu$ l and at pH 7.5. After incubation at room temperature for 30 minutes, the solution was filtered under vacuum through Whatman glass fibre filters presoaked for > 1 hour in 0.3% polyethyleneimine in buffer. The filters were then rapidly washed with 3 $\times$ 2ml of ice cold buffer and placed in 10 ml of scintillant (LKB, Optiphase Safe) before radioactivity was determined using a Packard Tri-Carb scintillation counter with pre-set windows for tritium. The counting efficiency was 35%.



The next section details some of the preliminary experiments to determine the best methods for preparing membranes, for separating bound ligand from free ligand and to determine the best conditions for the binding assay.

## The Binding Assay

### Choice of tubes for the binding assays

Plastic tubes were chosen for use in assays and for storage of materials since they are cheap, disposable and reasonably clean as supplied. The main choice to make was between polypropylene and polystyrene tubes with respect to the adsorption of  $^3\text{H}(-)$ nicotine to the plastic during experiments. To test this, a known amount of  $^3\text{H}(-)$ nicotine (220,000 dpm) in phosphate buffer pH 7.5 was added to two 3 ml polystyrene tubes (Sarstedt) and two 1.5 ml conical bottom polypropylene tubes (Sarstedt) and aliquots taken at time intervals after addition of the radioactive solution (1, 15, 30, 45 and 60 minutes). The amount of radioactivity in each aliquot was then measured and compared with the expected amount determined from the stock  $^3\text{H}(-)$ nicotine. Radioactivity associated with the plastic was extracted for 1 hour in 50 mM HCl (to cover the maximum level in the tubes reached by the radioactive solution).

The results were expressed as a percentage of the amount of radioactivity added to the tube at time zero. At 1 minute after addition,

102% and 98% of the radioactivity added to the polypropylene tubes was recovered: for the polystyrene tubes the values were 90% and 92%. Similar values were measured for the other time periods tested in each case. The acid extractable radioactivity for all four tubes was approximately 0.05% of the radioactivity originally added to the tubes. Since there appeared to be little adsorption of the  $^3\text{H}(-)\text{nicotine}$  to the polypropylene, this type of plastic tube was used in subsequent binding assays (incubation time typically less than 60 minutes) and for storage of the stock  $^3\text{H}(-)\text{nicotine}$ .

#### Choice of filter for use in the separation of bound from free ligand.

The choice of filter to use in the filtration assay was essentially between several types of Whatman glass fibre (GF) filter. The differences are in particle exclusion size of the pores and in the thickness of the filter, as detailed in table 5. Preliminary tests showed that there was little difference in measurements of specific binding using GF/B, GF/C or GF/F filters (this can be different, for example see Dawson, 1984), thus the important criteria in these experiments was the time taken for the filtration and wash procedure, the faster the better (to reduce dissociation of bound ligand). This was tested using a variety of wash volumes (from a dispenser) and with an independent assessment of the time taken. It was clear from these tests that the GF/C filter was the best to use in future experiments.

Table 5. Characteristics of Whatman glass fibre filters.

Filter	thickness (mm)	exclusion limit (µm)	filtration time (sec) for 3x3ml (n=1)
GF/A	0.31	1.6	1.7
GF/B	0.75	1.0	2.8
GF/C	0.31	1.2	1.7
GF/F	0.45	0.7	3.5

the thickness was determined on dry filters using a micrometer

Pretreatment of filters with polyethyleneimine (PEI).

The electrostatic charge on a wet glass fibre filter may affect the separation of bound from free ligand by influencing the amount and nature of receptor which is retained on the filter and by influencing the amount of ligand which binds to the filter alone. Glass fibre filters are likely to have a net negative charge to which the nicotinium ion (and acidic proteins) in these experiments would be attracted, thus overestimating the amount of nicotine bound to receptor. Other studies on the receptors for <sup>3</sup>H(-)nicotine in brain have used polylysine or PEI treatment of the filters (e.g. Marks & Collins, 1982).

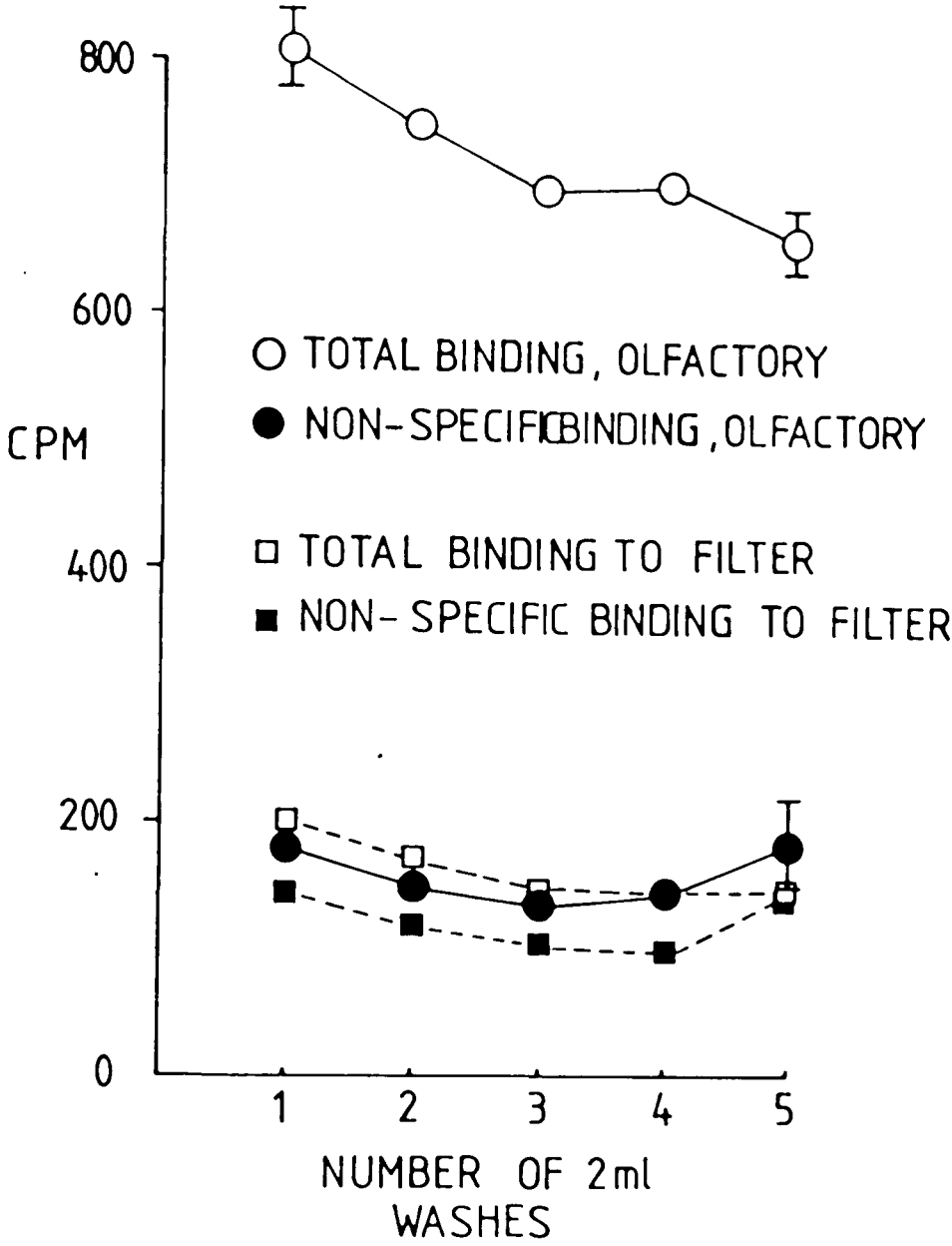
A preliminary study showed that the amount of <sup>3</sup>H(-)nicotine binding to PEI treated filters was half that found binding to filters treated with buffer only (tested at pH 6, 7.5 and 8). PEI treatment of the filters was used in all other experiments in order to reduce any overestimate of the nicotine bound to the receptor (the nature of the nicotine binding sites in

olfactory preparations was not known, thus PEI treatment would also facilitate retention of all possible binding components on the filter). In later experiments at optimal conditions for binding it became clear that the amount of  $^3\text{H}(-)\text{nicotine}$  binding to the filter was insignificant compared with the amount of ligand associated with the olfactory binding sites.

#### Number of washes of the filter

It is important to determine the volume and number of washes of the filter following the initial separation, in order to determine accurately the amount of ligand bound to the receptors. Too much washing may result in dissociation of bound ligand from the receptor whereas too little washing may not separate ligand free in solution and ligand bound non-specifically to the filter and other sites in the preparation from that bound to the receptor. Results from several experiments suggested that a 2ml wash would pass rapidly through the filter and using increasing numbers of washes, gave good separation of bound and free ligand. The incubation volume of the binding assay was typically 250 $\mu\text{l}$  in preliminary experiments and 200 $\mu\text{l}$  in the final series of experiments. A damp GF/C filter under filtration assay conditions holds  $59.6 \pm 6.8 \mu\text{l}$  of liquid (mean  $\pm$  s.d., estimated by increase in weight of five dry samples), thus a 2ml wash is in 30 fold excess of this and should be a sufficient volume for effective washing.

Figure 18. Effect of Increasing Filter Washing on Bound  $^3\text{H}(-)\text{Nicotine}$



The data points for olfactory tissue show the mean  $\pm$  standard deviation (included within a point if not illustrated) for triplicate determinations. The values for filter binding alone are means of duplicate determinations. Data is from a single experiment.

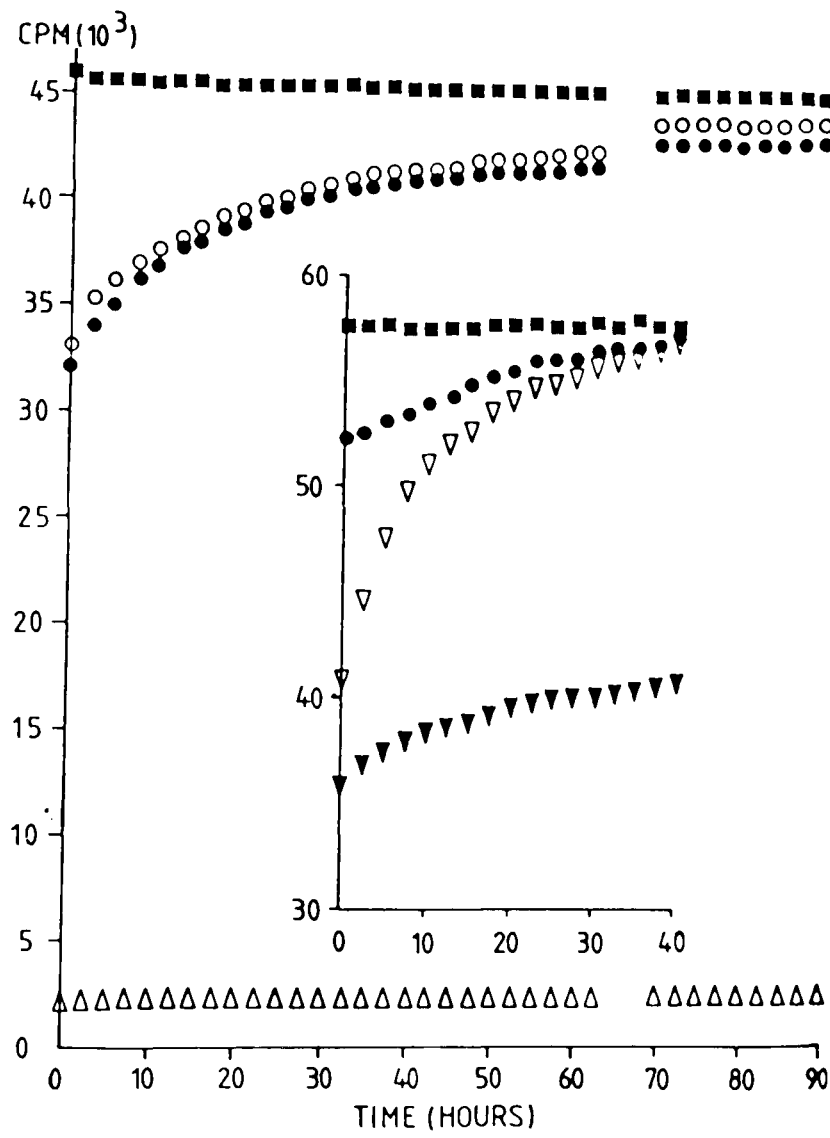
To estimate the number of 2ml washes required for separation of bound and free ligand, triplicate determinations of the total and the non-specific radioactivity bound to an olfactory preparation and to the filters alone was made for 1, 2, 3, 4, and 5  $\times$  2ml washes of the filter. The results

(figure 18) show that good separation of bound and free is achieved even after a 1x2ml wash, but that better replicates are measured with more washes. The amount of specifically bound  $^3\text{H}(-)\text{nicotine}$  is affected slightly by a larger number of washes but does not decrease dramatically, suggesting that dissociation of bound ligand does not occur to any great extent during the filtration and wash procedure. In subsequent experiments a 3x2ml wash was used. This will give good separation of bound and free  $^3\text{H}(-)\text{nicotine}$  in a rapid wash procedure (filtration and wash of a single incubation were typically complete within 5 seconds).

#### Measurement of the radioactivity on the filters

In order to measure the amount of  $^3\text{H}(-)\text{nicotine}$  binding to the olfactory preparation the radioactivity on the filters must be extracted into scintillant and measured in a liquid scintillation counter. Other workers have suggested that the filters from binding assays should be dried before scintillant is added (e.g. Galper *et al.*, 1977) but this poses an important question. Has all the radioactivity been extracted from the filters by the scintillant or does some remain on or within the filter? If so, the amount of radioactivity will be underestimated since the energy transfer process from the tritium label to the sensitive chemicals in the scintillant will be reduced. This has been demonstrated to be the case when using  $^3\text{H}$ -QNB in receptor binding studies (Dawson, 1984). In addition it is possible that radiolabelled odorants may evaporate from the filter during the drying procedure.

Figure 19. Extraction of Radioactivity Associated with the Filter into Scintillant



All values are the means of duplicate determinations (within 10%) from single experiments (main figure and inset) and were measured under refrigerated conditions. The break in the data on the main figure represents an 8 hour period at room temperature. The volume of scintillant (S) was 10 ml. Filters (F) were presoaked in 0.3% PEI before use. Operations were carried out in the described sequence at time zero (T - Tritium label);

■ S + T, add damp F; ● S, add damp F + T; ○ S, add damp F + T + protein  
△ S, add air dried F + T; ▼ F + T + 1ml water, add S; ▽ F + T + 1ml 0.2M NaOH, add S

legend continues

In an experiment using damp F + T in vials kept at room temperature or refrigerated before scintillation counting, measurements at time = 0, 16, 24, and 48 hours gave the following results (expressed as a percentage of added cpm). Room temperature - 62, 92, 98, 99% ; refrigerated - 58, 79, 86, 90%. In subsequent experiments samples were left at room temperature for 24 hours before scintillation counting.

The results in figure 19 show that drying the filters before adding the scintillant is not to be recommended. The apparent lack of radioactivity on the dried filters suggests that the  $^3\text{H}(-)\text{nicotine}$  may in fact evaporate during the drying procedure. After 60 hours, >90% of the radioactivity had been extracted from the damp filters and there was no apparent effect of protein on this extraction. Liquid scintillation counting was performed in a refrigerated chamber which is likely to reduce the time taken for the radioactivity to be extracted from the filter, other results (see legend to figure 19) suggest that the maximum radioactivity is measured when the vials (plastic) are first left for 24 hours at room temperature. The addition of NaOH to the damp filters does not improve the extraction rate and water alone appears to prevent the release of the maximum amount of radioactivity. The conclusion from these experiments must be that the scintillant is best added to the damp filters and as soon as possible after the filtration and wash procedure is complete.



### Centrifugation binding assay

Preliminary experiments showed that specific binding of  $^3\text{H}(-)\text{nicotine}$  to olfactory membranes could be measured by this technique (using an MSE bench centrifuge at the high speed setting and a Sorvall RC-2B centrifuge at  $18,000\times g$ ). However, the values measured were smaller than those observed using the filtration assay, showed poor replicability and were subject to very high levels of non-specific binding. The effectiveness of the centrifugation in precipitating all of the binding component must also be questioned since the size of the membrane vesicles prepared from olfactory tissue by sonication is not known. This assay was not investigated further.

### Preparation of Olfactory Membranes

#### Sonication of the olfactory turbinates

Since the effect of sonication on the cells of the olfactory epithelium is not fully understood, the minimum sonication time necessary to obtain a preparation to which  $^3\text{H}(-)\text{nicotine}$  binds was selected. The olfactory turbinates from three rats were removed and treated as described in materials and methods. The turbinates from one rat were sonicated for 5 seconds, the second for 20 seconds and the third for 40 seconds, after which the  $1000\times g$  supernatant from each was prepared. Duplicate measurements of total binding and non-specific binding of  $^3\text{H}(-)\text{nicotine}$  to

the preparation from each sonication time were then taken. The results of this experiment are shown below in table 6.

Table 6. Duration of sonication time on olfactory turbinates and the effect on the binding of  $^3\text{H}(-)$ nicotine to olfactory membranes.

sonication time (sec)	protein in 1000×g supernatant (mg/ml)	mean cpm specifically bound per mg protein
5	1.55	4526
20	2.00	3343
40	3.70	3167

(mean values are from duplicate determinations within 20%).

The preparation with the most activity resulted from the 5 second sonication of the olfactory turbinates. Since sonication for 10 seconds seemed to be too long, I decided that a sonication time of 8 seconds would be the best to use in future experiments. This sonication would be long enough to remove the binding component from the turbinates yet short enough to minimise any damage to the membranes. Other work in this laboratory has shown that a sonication time of 5 seconds causes the release of most of the odorant-modulated adenylate cyclase activity (Dr. S.G. Shirley, unpublished result).

### Effect of buffer composition and of ions on the binding

The effect of buffer strength and composition was investigated in one experiment using four different buffers for the preparation of membranes (sonication time of the olfactory turbinates was the same for each). Two buffers were investigated, phosphate-saline and HEPES-saline and at two concentrations, 5mM and 50mM (saline in all cases at 0.9% NaCl w/v). It was desirable to keep the buffer as simple as possible since the effect of organic buffers on the olfactory receptors is not known. Sucrose was not used in the preparations since it is known to stimulate olfactory adenylate cyclase activity (Shirley *et al.*, 1986). The effect of adding sodium, calcium, magnesium and EGTA to the binding assay was also studied. Results from duplicate determinations are shown in table 7.

These results suggested that preparation of the olfactory membranes and binding of  $^3\text{H}(-)\text{nicotine}$  was better in phosphate buffer than HEPES buffer and that high concentrations of phosphate reduced the amount of specific binding measured. This effect of phosphate concentration on the binding was also seen in other experiments (not shown). Although the final concentration of the ions added to the binding assay was not determined, the concentrations added were such that large effects on the binding should be seen. Neither calcium, magnesium nor EGTA had large effects on the binding in this experiment. An increase in ionic strength of the binding buffer reduced the amount of specific binding measured.

Table 7. Effect of buffer strength and of ions on the binding of  $^3\text{H}(-)\text{nicotine}$  to olfactory membranes (NaCl at 0.9%).

preparation and binding buffer	mean cpm specifically bound / mg protein	mean cpm non-specifically bound / mg protein
5mM phosphate/NaCl	3191	335
50mM phosphate/NaCl	2516	261
5mM HEPES/NaCl	1306	441
50mM HEPES/NaCl	2351	249
ions added to 5mM phosphate/NaCl binding buffer		
50 $\mu\text{M}$ $\text{Ca}^{2+}$	2733	266
1mM $\text{Mg}^{2+}$	3338	255
300mM $\text{Na}^+$	1742	202
10mM EGTA	2724	242

(mean values are from duplicate determinations within 10%).

In another experiment the effect of Phosphate, HEPES or TRIS buffer on the preparation and binding of  $^3\text{H}(-)\text{nicotine}$  to olfactory membranes was tested. The results of this experiment (table 8) showed that a 5mM phosphate/0.9% NaCl buffer at pH 7.5 gave results as good as for any other buffer tested. The replicability of results within an experiment (using the filtration binding assay) is also shown in table 8. The effect on the binding of borate (adjusted to pH 7.5 before addition to the assay) was

also investigated. Addition of 1mM and 5mM (final concentration)  $H_3BO_3$  to the 5mM phosphate/0.9% NaCl binding buffer caused a 20% increase in the amount of specifically bound nicotine measured, without increasing non-specifically bound nicotine. The increase in specific binding of  $^3H(-)$ nicotine by borate may be explained by a pH effect on the ligand (see later) and was not investigated further.

Table 8. Effect of phosphate, TRIS, HEPES and of Borate on the binding of  $^3H(-)$ nicotine to olfactory membranes.

buffer/0.9% NaCl (pH7.5)	cpm bound per $\mu$ g protein		
	total	non-specific	specific
50 mM HEPES	1898 $\pm$ 127	214 $\pm$ 15	1684
50 mM TRIS	3545 $\pm$ 218	361 $\pm$ 63	3184
5 mM phosphate	3493 $\pm$ 105	432 $\pm$ 117	3061
borate in phosphate binding buffer			
1 mM	4126 $\pm$ 170	401 $\pm$ 92	3725
5 mM	4389 $\pm$ 540	427 $\pm$ 93	3962

results are mean  $\pm$  standard deviation of triplicate determinations

pKa of the buffers at 25°C (Dawson et al., 1986)

HEPES 7.5; TRIS 8.1; Phosphoric acid 6.8 (2nd protonation)

**Effect of pH on the preparation of membranes.**

Preliminary experiments showed that preparation of membranes and binding in 50 mM phosphate/0.9% NaCl buffer at pH 7.8 gave less specifically bound cpm per mg protein than for the comparable experiment using 5mM phosphate/0.9% NaCl at pH 7.5. Preparation of membranes in 5mM phosphate/0.9% NaCl at pH 7.5 and subsequent binding in 28 mM phosphate/0.9% NaCl at pH 7.8 gave more binding than in 28 mM phosphate/0.9% NaCl at pH 7.5, using the same preparation. The reduced binding in the preparation made at pH 7.8 may be explained by the aggregation and precipitation of the binding component during the centrifugation step. Membranes were prepared in 5 mM phosphate/0.9% NaCl pH 7.6 in all subsequent experiments.

## Chapter 4.

### Characterisation of Nicotine Binding Sites in Rat Olfactory and Respiratory Epithelia.

#### Introduction

Interest in nicotine centres around its pharmacological effects as an agonist for the nicotinic acetylcholine receptor and the accompanying physiological effects of tobacco smoke, of which nicotine is an active ingredient (Hall, 1970; Mangan & Golding, 1984; Benowitz, 1986). It has been shown that nicotine binds specifically to membrane preparations from rat brain (Abood *et al.*, 1985b; Romano & Goldstein, 1980; Sloan *et al.*, 1984; Lippiello & Fernandes, 1986), mouse brain (Marks & Collins, 1982; Sershen *et al.*, 1981) and human brain (Shimohama *et al.*, 1985). There is good evidence that this brain receptor is not equivalent to the ganglionic or neuromuscular nicotinic acetylcholine receptor (e.g. Marks *et al.*, 1986; Wonnacott, 1986; Kemp & Morley, 1986; Collins *et al.*, 1986). Nicotine also binds to non-cholinergic sites in human leucocyte membranes (Hoss *et al.*, 1986) and in hepatocyte membranes (Abood *et al.*, 1985a).

Pyridine derivatives are thought to be important flavour components of many products such as tea, coffee and tobacco (Vernin, 1982). Nicotine is believed to be one such component in tobacco smoke (Enzell, 1981).

Electrophysiological recordings (chapters 1 and 2) show that nicotine vapour stimulates an *in vitro* olfactory preparation in a manner similar to other odorants, suggesting that there are olfactory receptors which respond to nicotine and that nicotine is an odorant for the rat. These results prompted me to study the interaction of radiolabelled nicotine with membranes prepared from rat olfactory epithelium. The preparation used was similar to the one described by Shirley *et al.* (1986) which contained odorant modulated adenylate cyclase activity. An odorant modulated olfactory adenylate cyclase in both the frog and the rat has also been demonstrated by other workers (Pace *et al.*, 1985; Sklar *et al.*, 1986).

There have been several attempts to identify and isolate an olfactory receptor by using the binding of a radiolabelled odorant as a marker. These include binding of camphor (Fesenko *et al.*, 1979), 2-i-bu-3-methoxypyrazine (Wood & Dodd, 1984; Pelosi *et al.*, 1982; Pevsner *et al.*, 1985), androstenone (Dodd & Persaud, 1981; Gennings *et al.*, 1977) and anisole (Price, 1978) in mammalian nasal mucosa and amino acids in fish (Rhein & Cagan, 1980). Binding of a benzodiazepine to olfactory neurons has also been demonstrated (Anholt *et al.*, 1984) as has the binding of the muscarinic antagonist quinuclidinyl benzilate to a site in the olfactory mucosa of the salamander (Hedlund & Shepherd, 1983).



The metabolism of nicotine by hepatic enzymes and cytochrome P-450 in particular has been studied in detail elsewhere (e.g. McCoy *et al.*, 1986; Nakayama *et al.*, 1985; Abood *et al.*, 1985a). Since there are high levels of these metabolic enzymes in olfactory epithelium (Hadley & Dahl, 1982; Bond, 1983; Reed *et al.*, 1986; Jenner & Dodd, 1988), the possibility that nicotine interacts with these enzymes is also considered.

## Materials and Methods

### Chemicals

The suppliers of  $^3\text{H}(-)$ nicotine and  $\text{S}(-)$ Nicotine and the details on the storage, purification and purity are given in chapter 3 and appendix A. Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone; see figure 20) was purchased from Aldrich and  $\alpha$ -Bungarotoxin from Sigma. All other chemicals used were of the highest quality and were used without further purification. (+)Nicotine bitartrate was a gift from Dr. R.B. Barlow, Department of Pharmacology, University of Bristol, U.K. and dihydro- $\beta$ -erythroidine was a gift from Mr. R.G. Benfield, Merck, Sharp and Dohme Development Laboratories, Herts., U.K.

### Protein assay

Protein was assayed by the method described by Hartree (1972) using bovine serum albumin as standard (see chapter 3).

**Olfactory membrane preparation**

The method used was essentially as described in chapter 3. The ethmoturbينات were carefully removed from a freshly killed male Wistar rat (275-300 g) and placed in 10 volumes of ice cold phosphate (5 mM)/NaCl (0.9%)/EGTA (1 mM) buffer, pH 7.6. After careful washing in two changes of this buffer and one of buffer minus EGTA, the ethmoturbينات (typically 100 mg wet weight per animal) were placed in 0.7 ml of buffer without EGTA and sonicated for 8 seconds. The liquid was then decanted and the turbينات rinsed in a further 0.3 ml of buffer, after which the fractions were pooled and centrifuged at 1000×g for 15 minutes at 4°C. The supernatant was used without further treatment. For comparison in these studies, tissue anterior to the olfactory turbينات was taken from the nasal cavity and treated as above. This tissue, defined here as respiratory, was taken from the same region from which it was not possible to record an EOG to nicotine vapour (see chapter 1).

**<sup>3</sup>H(-)Nicotine binding assay**

Binding assays were performed in polypropylene tubes containing <sup>3</sup>H(-)nicotine, sonicated material (0.1-0.2 mg protein) and phosphate-saline buffer (final concentration 17 mM phosphate/0.9% NaCl) to a final volume of 0.2 ml at pH 8.4. Non-specific binding was determined by including 1 mM nicotine in the assay. Incubations were started by the addition of protein at the appropriate temperature for the experiment. The tubes were incubated for 15 minutes at 20°C then the assay mix was filtered under vacuum through Whatman GF/C filters (presoaked for 1 hour in 0.3%

polyethyleneimine) and rapidly washed with  $3 \times 2$  ml of ice cold buffer. The filtration apparatus was precooled on ice before use, as were the filters which were also rinsed with 2 ml of ice cold buffer immediately before the solution was filtered. The filters were then placed in 10 ml of scintillant and left for 24 hours before liquid scintillation counting at 35% efficiency.

### Saturation Studies

To attain high concentrations,  $^3\text{H}(-)\text{nicotine}$  was diluted with unlabelled  $(-)\text{nicotine}$  to 4 Ci/mmol and increasing amounts added to the assay tubes (0.2-2.5  $\mu\text{M}$  final concentration). The radioactivity retained on the filter was taken to represent the ligand bound to receptor and was analysed accordingly (the value for "total" ligand in each assay was taken as the concentration thought to be added to the tube, experimental measurement of "total" ligand was impractical and did not give markedly different values from the theoretical value).  $K_D$  and  $B_{\text{max}}$  values were determined from least squares linear regression analysis of Scatchard plots from the data (see figure 15).

### Other Studies

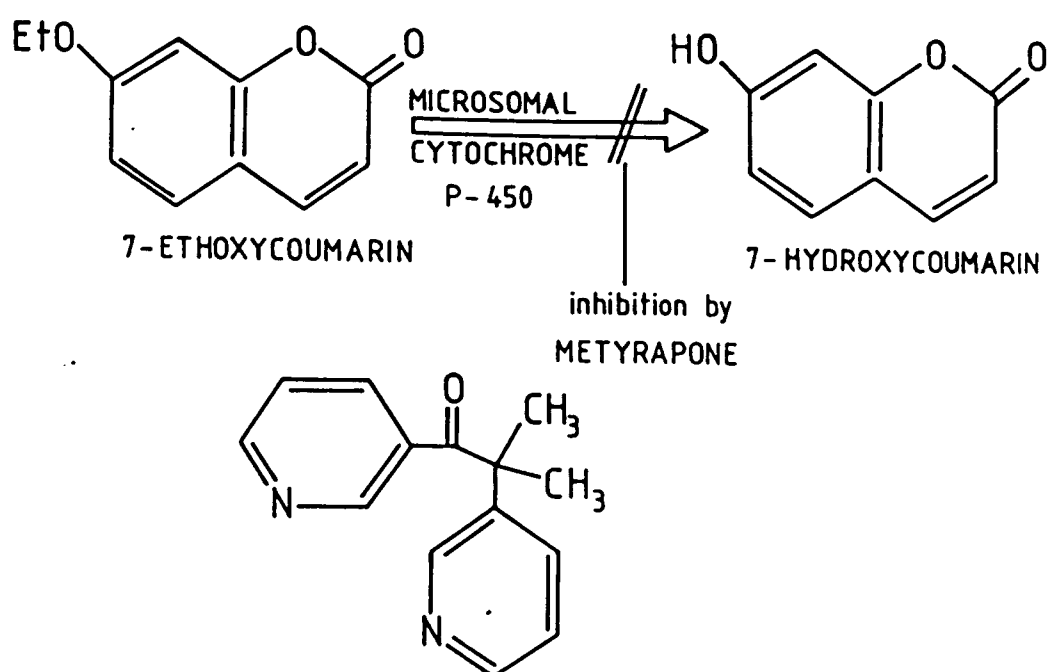
In these studies  $^3\text{H}(-)\text{nicotine}$  (full specific activity) at 19 nM final concentration was used.  $\text{IC}_{50}$  values (concentration of inhibitor necessary for 50% inhibition of binding) were determined by Hill plot analysis and by a method described by Bylund (1986). Lines were fitted to the data by least squares linear regression.

### Identification of bound compound

Bound compound was extracted from the filters and analysed by thin layer chromatography by an adaption of the procedure used by Marks & Collins (1982). Ten filters from a typical experiment to measure total bound ligand were cut into small pieces and placed into 2 ml of 0.2 M NaOH. Other filters were measured for radioactivity as normal and showed the specifically bound ligand to be 83% of the total (therefore, non-specifically bound  $^3\text{H}(-)\text{nicotine}$  was not extracted separately). 100 n moles of  $(-)\text{nicotine}$  were added as carrier and the filters homogenised by hand. The liquid was decanted into 4 ml of ethyl acetate (analytical grade) and the filters re-homogenised in a further 1 ml of 0.2 M NaOH which was then added to a second tube containing 4 ml of ethyl acetate. The  $^3\text{H}(-)\text{compound}$  was extracted into the organic layer by repeated inversion of the tubes, which were then left (sealed) for 1 hour at room temperature. Parallel tubes containing  $^3\text{H}(-)\text{nicotine}$  were extracted in the same way during the experiment. The tubes were then centrifuged at 1000 rpm for ten minutes and the organic layers decanted. The ethyl acetate extracts from the filters were pooled and evaporated to dryness under a gentle stream of nitrogen gas. The extent of evaporation of the ligand was not known. The extracted ligand was then dissolved in 200  $\mu\text{l}$  of methanol for use in t.l.c. experiments.

### 7-Ethoxycoumarin de-ethylase activity

Figure 20. Fluorescence Assay for Cytochrome P-450 and Inhibition of the Enzyme by Metyrapone



The de-ethylation of 7-ethoxycoumarin (figure 20), a substrate for nasal cytochrome P-450-dependent enzymes (Reed et al., 1986; Jenner & Dodd, 1988), was assayed as follows (adapted from a procedure used by Dr. J. Jenner, this laboratory). This is a fixed-point assay at one concentration of substrate and at pH 7.6.

The incubation consisted of 0.1 ml of 1M Tris-HCl at pH 7.6, 0.1 ml of a freshly made NADPH generating system (5mM NADP, 60 mM glucose-6-phosphate and 10 units per ml of glucose-6-phosphate dehydrogenase, made up in 25mM MgCl<sub>2</sub>) and 0.7 ml containing the sample, any inhibitor and distilled water, all done in duplicate. The tubes (thoroughly cleaned glass tubes with screw tops, suitable for use in a centrifuge at 1000 rpm) were

then pre-incubated at 37°C for 10 minutes, after which the reaction was started by the addition of 0.1 ml of substrate (7-ethoxycoumarin, final concentration 10  $\mu$ M). After 20 minutes the reaction was terminated by the addition of 0.5 ml of 0.5M glycine-trichloroacetic acid buffer pH 2.2 (pH adjusted with 50% TCA).

The substrate, 7-ethoxycoumarin, is particularly difficult to dissolve in water and should be prepared several hours before the experiment by alternate heating at 50 °C and vigorous stirring. Interestingly, 7-ethoxycoumarin in solution has a distinctive almost aniseed-like odour (my description). A standard line was also determined in the experiment using 7-hydroxycoumarin, the fluorescent product of the de-ethylation of 7-ethoxycoumarin by cytochrome P-450 (figure 20). A freshly made 100 mM stock solution in ethanol should be diluted in water to 100  $\mu$ M. Standards for the experiment were made from this 100  $\mu$ M stock, and additions of 0.1 ml made to the tubes in the place of substrate. A standard line was determined for 0.5, 1, 2, 3 and 4  $\mu$ M final concentration of 7-hydroxycoumarin. Blanks were determined by heating the sample at 60 °C for 30 minutes before assaying (when using a microsomal preparation, the blanks consist of all components minus the NADPH generator). The amount of protein added to the tubes was typically between 0.05 and 0.08 mg for the 1000 $\times$ g olfactory supernatant.

7-Hydroxycoumarin was extracted (repeated inversion of the tubes) from the reaction mixture with 6 ml of 60-80 hexane for 10 minutes

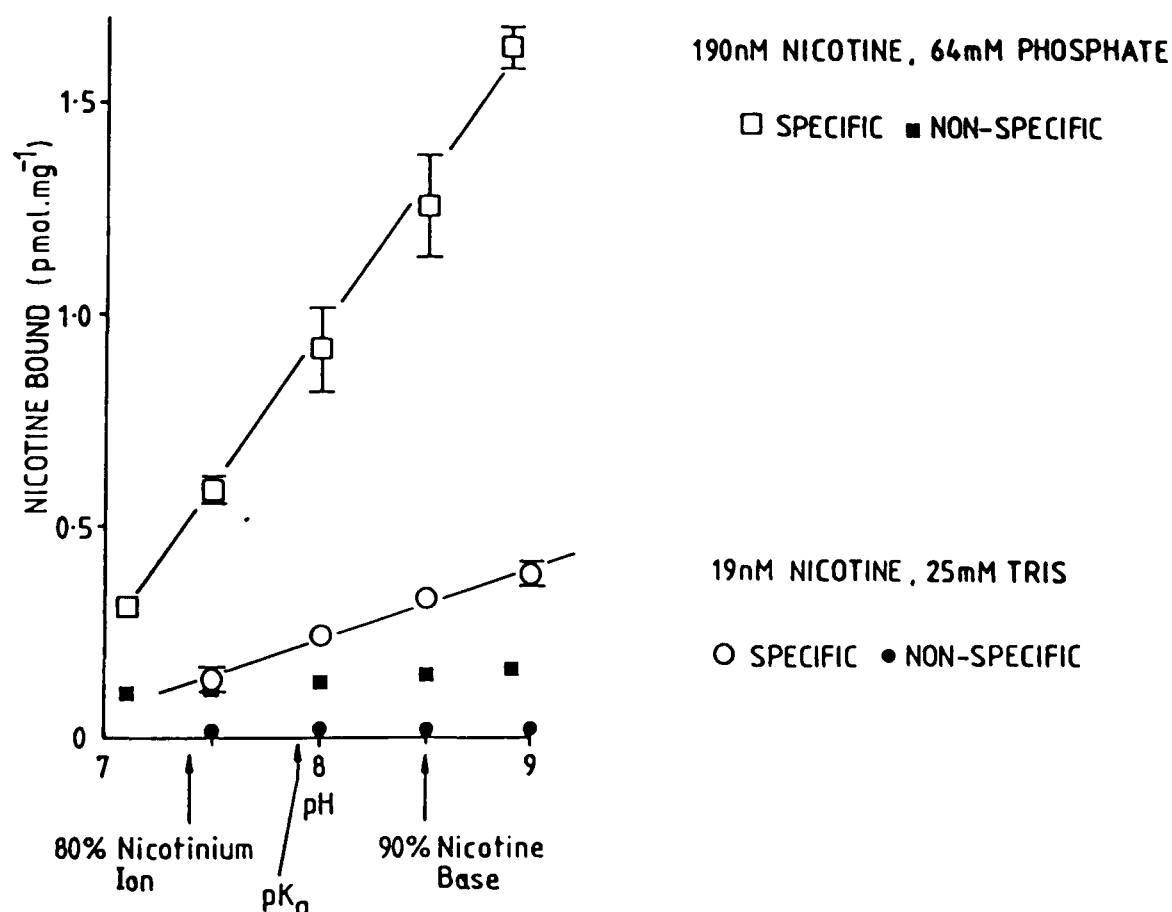
followed by centrifugation for 20 minutes at 1000 rpm. The aqueous layer was then frozen in a dry ice-methanol freezing bath and the organic layer discarded. The thawed aqueous layer was then extracted for 10 minutes with 4 ml of diethyl ether (washed before use twice with twice the volume of 0.2 M glycine-NaOH pH 10.6; pH adjusted with 10M NaOH), centrifuged and 2.5 ml of the organic layer pipetted into fresh tubes. This 2.5 ml aliquot was then extracted with 5 ml of 0.2 M glycine-NaOH pH 10.6 for 10 minutes, centrifuged and the aqueous layer separated from the organic layer by freezing in the dry ice-methanol. The aqueous layer could then be left overnight in the cold room before the next step. The tubes were heated to 50 °C for 2-3 hours to drive off included ether after which the liquid was transferred to plastic cuvettes. Fluorescence was determined in a Perkin-Elmer MPF-3 spectrofluorimeter at excitatory wavelength 370 nm and emission wavelength 450 nm. Data for the standard line and samples were calculated by linear regression analysis.

## Results

### Effect of pH on $^3\text{H}(-)$ nicotine Binding

The binding of  $^3\text{H}(-)$ nicotine was strongly affected by the pH of the assay buffer (figure 21). Both specific and non-specifically bound  $^3\text{H}(-)$ nicotine were linear with pH as measured at two concentrations of nicotine and in two different buffer solutions.

Figure 21. Effect of Change in pH on  $^3\text{H}$ (-)-Nicotine Binding to Olfactory Membranes



Specific binding values show mean  $\pm$  standard deviation (n=3), non-specific binding values are means only (n=2) from single typical experiments at each condition.

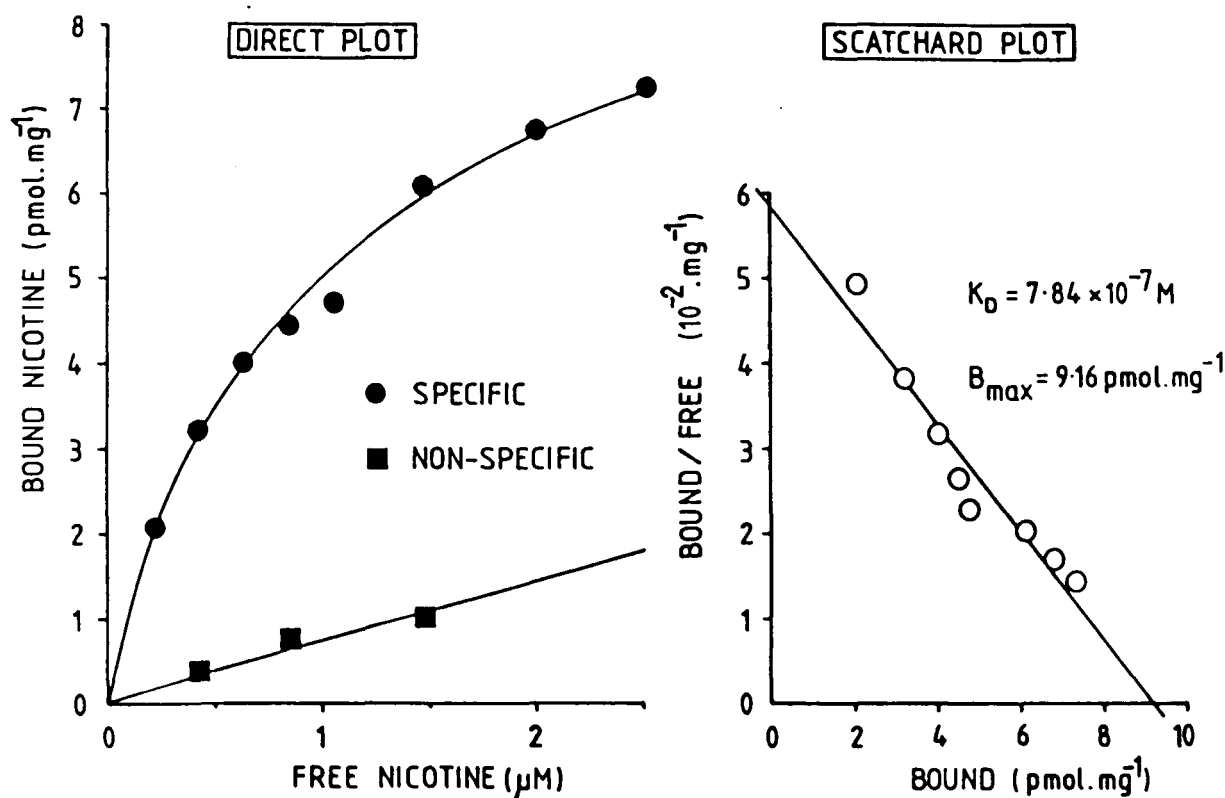
That nicotine binds to a greater degree at high pH's suggested that the unprotonated form of nicotine ( $\text{pK}_a=7.9$ , see figure 2) was involved in the interaction with the binding site, though the continued increase in binding at the higher pH's could not be explained in this way. Non-specific binding of  $^3\text{H}$ (-)-nicotine did not increase significantly with increasing pH. This observation helped a great deal in setting up the



next phase of experiments since more bound radioactivity could be measured and errors would be minimised. It was felt necessary to continue using the phosphate buffer at as low a concentration as possible, consequently binding assays were conducted at pH 8.4 (final pH after addition of preparation in 5 mM phosphate/0.9% NaCl at pH 7.6 to binding assay buffer of 25 mM phosphate/0.9% NaCl at pH 8.7.). Use of a 25 mM Tris/0.9% NaCl buffer at pH 8.4 in later experiments gave results as good as, if not better than those measured using the phosphate buffer, suggesting that the assay may be further improved.

#### Binding parameters for $^3\text{H}(-)\text{nicotine}$

Figure 22. Binding of Nicotine to Olfactory Membranes



The data points in the Direct plot are mean values (duplicates within 8%) from a single experiment at pH 8.4.

$^3\text{H}(-)\text{Nicotine}$  bound specifically to the crude olfactory membrane preparation (figure 22) over the range of concentrations of nicotine tested. Scatchard analysis of this data (figure 22) suggested a single binding site with  $K_D=784$  nM and  $B_{\text{max}}=9.16$  pmol bound per mg protein. A duplicate experiment gave  $K_D=606$  nM and  $B_{\text{max}}=7.33$  pmol bound per mg protein. These two experiments were conducted at pH 8.4 in a 17 mM phosphate/0.9% NaCl buffer.

One experiment in 5 mM phosphate/0.9% NaCl pH 7.5 and using nicotine over a final concentration range from 20 to 260 nM gave binding parameters of  $K_D=580$  nM and  $B_{\text{max}}=4.3$  pmol bound per mg protein. Measurements, in duplicate, did not differ by more than 15%.

Specific binding, determined by subtracting non-specific binding (measured in the presence of 1 mM unlabelled nicotine; similar results were obtained at 0.1 mM unlabelled nicotine) from total binding, was routinely 80% of the total. Non-specific binding was linear with increasing nicotine concentration in all experiments.

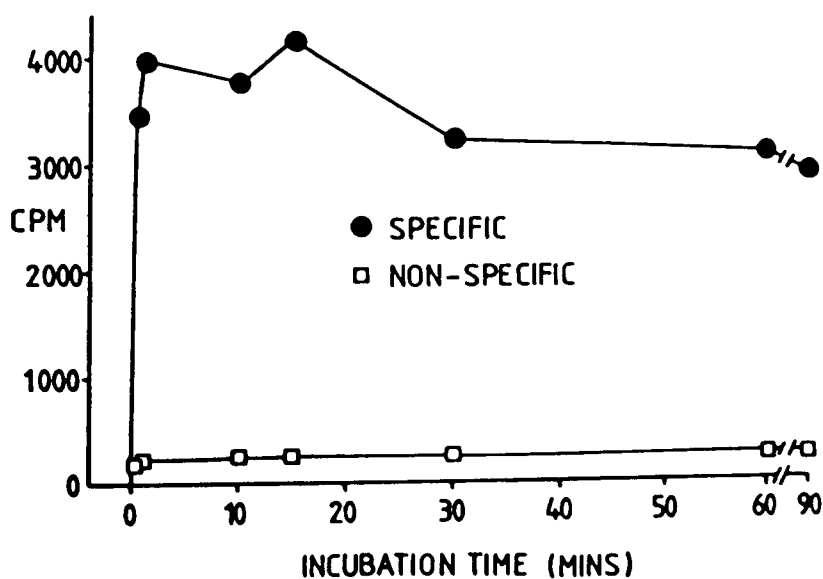
### Effect of increasing protein concentration

$^3\text{H}(-)\text{Nicotine}$  binding to the olfactory preparation was also linear with protein when tested at pH 7.4, 7.7 and 8.5, in either 25 mM Tris / 0.9% NaCl or 5 mM phosphate buffer (not shown).

### Association rate constant and its change with temperature

The association rate of  $^3\text{H}(-)\text{nicotine}$  with the olfactory preparation was extremely rapid at 20°C (figure 23) and even when measured on ice (not shown). It was not possible to determine an association rate constant from the data. Similar results were obtained at pH 7.6. Although equilibrium was attained within tens of seconds, all incubations were for 15 minutes, after which the reaction was stopped by filtration. Over a longer period of time (15-90 minutes) some loss of specifically bound  $^3\text{H}(-)\text{nicotine}$  was observed. Non-specifically bound  $^3\text{H}(-)\text{nicotine}$  was saturated in seconds at both temperatures.

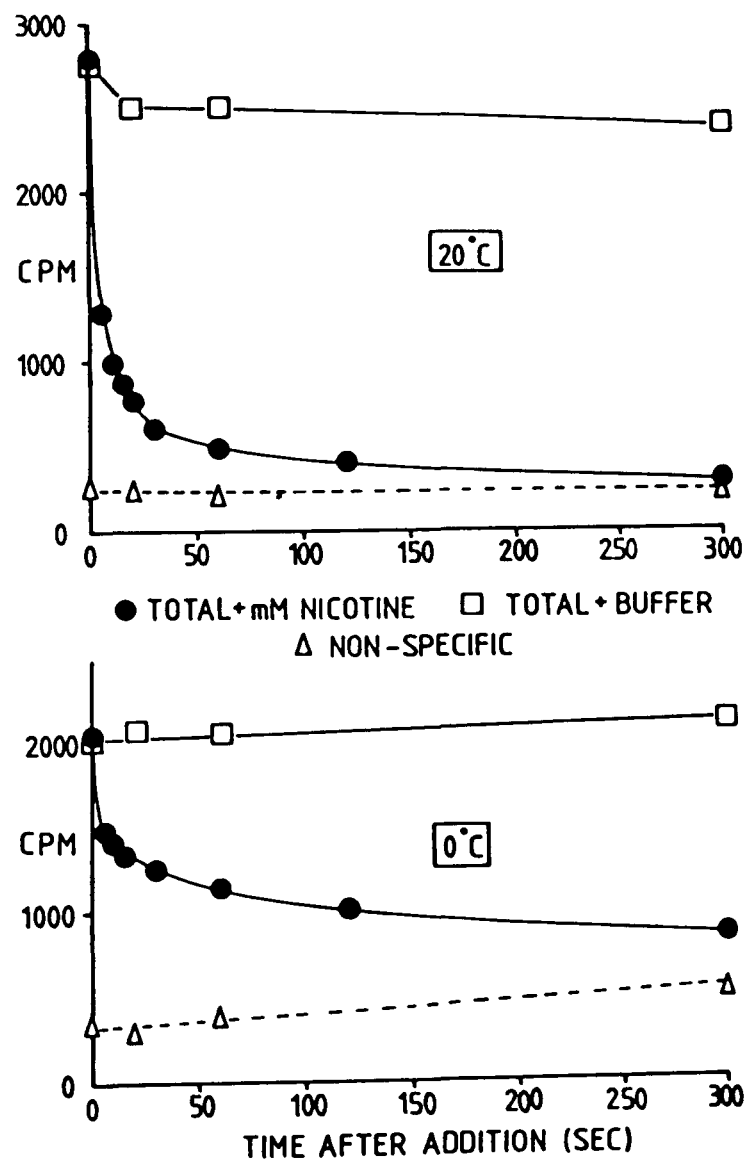
Figure 23.  $^3\text{H}(-)\text{Nicotine}$  Binding at 20°C Versus Incubation Time



The points show mean values ( $n=2$ ) from a single typical experiment (duplicates within 10%)

## Dissociation rate constant and its change with temperature

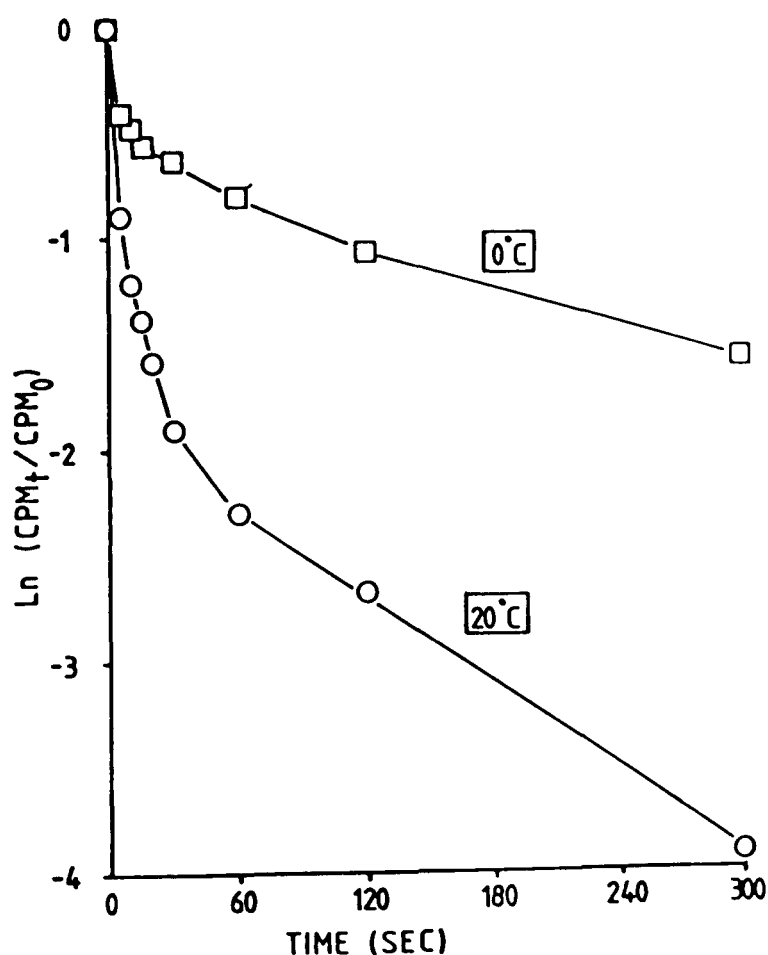
Figure 24. Dissociation of  $^3\text{H}$ (-)-Nicotine from Olfactory Membranes at  $20^\circ\text{C}$  and  $0^\circ\text{C}$



The points are mean values (duplicates within 10%) from a single representative experiment at each temperature. At time zero, 10  $\mu\text{l}$  of either unlabelled nicotine (to 1 mM) or of buffer alone was added to the assay solution (volume of 200  $\mu\text{l}$ ) at equilibrium. Samples were filtered at the given time intervals after this addition.

The dissociation of specifically bound  $^3\text{H}(-)$ nicotine from the binding site was also rapid and was also sensitive to a change in temperature (figures 24 and 25 ). At  $20^\circ\text{C}$  the dissociation of  $^3\text{H}(-)$ nicotine appears bi-phasic with a very fast phase ( $t_{1/2}=6$  seconds) and a much slower phase ( $t_{1/2}=101$  seconds). On ice the fast dissociation step was less apparent and the slower phase was reduced by a factor of 2 ( $t_{1/2}=206$  seconds).

Figure 25. Log Plot of Specifically Bound  $^3\text{H}(-)$ Nicotine with Time  
Following Addition of Excess Unlabelled Nicotine



The data has been calculated from that shown in figure 24.

at  $20^\circ\text{C}$ , rapid phase  $k'_{\text{off}} = 0.1224 \text{ s}^{-1}$ ,  $t_{1/2} = 6$  seconds

slower phase  $k'_{\text{off}} = 6.83 \times 10^{-3} \text{ s}^{-1}$ ,  $t_{1/2} = 101$  seconds

at  $0^\circ\text{C}$ , slower phase  $k'_{\text{off}} = 3.37 \times 10^{-3} \text{ s}^{-1}$ ,  $t_{1/2} = 206$  seconds

It was also observed that at the lower temperature (0°C) the maximum amount of specifically bound  $^3\text{H}(-)$ nicotine is typically half of the maximum amount measured at the higher temperature (20°C), all other conditions being equal. An increase in the amount of  $^3\text{H}(-)$ nicotine bound non-specifically at 0°C accounted for only a small fraction of this difference. Incubation of the reaction mix for 5 minutes at 20°C then 10 minutes at 0°C also gave the smaller amount of  $^3\text{H}(-)$ nicotine binding.

Similar results were observed at pH 7.6, although the smaller amount of  $^3\text{H}(-)$ nicotine binding made analysis of the results obtained at this pH difficult.

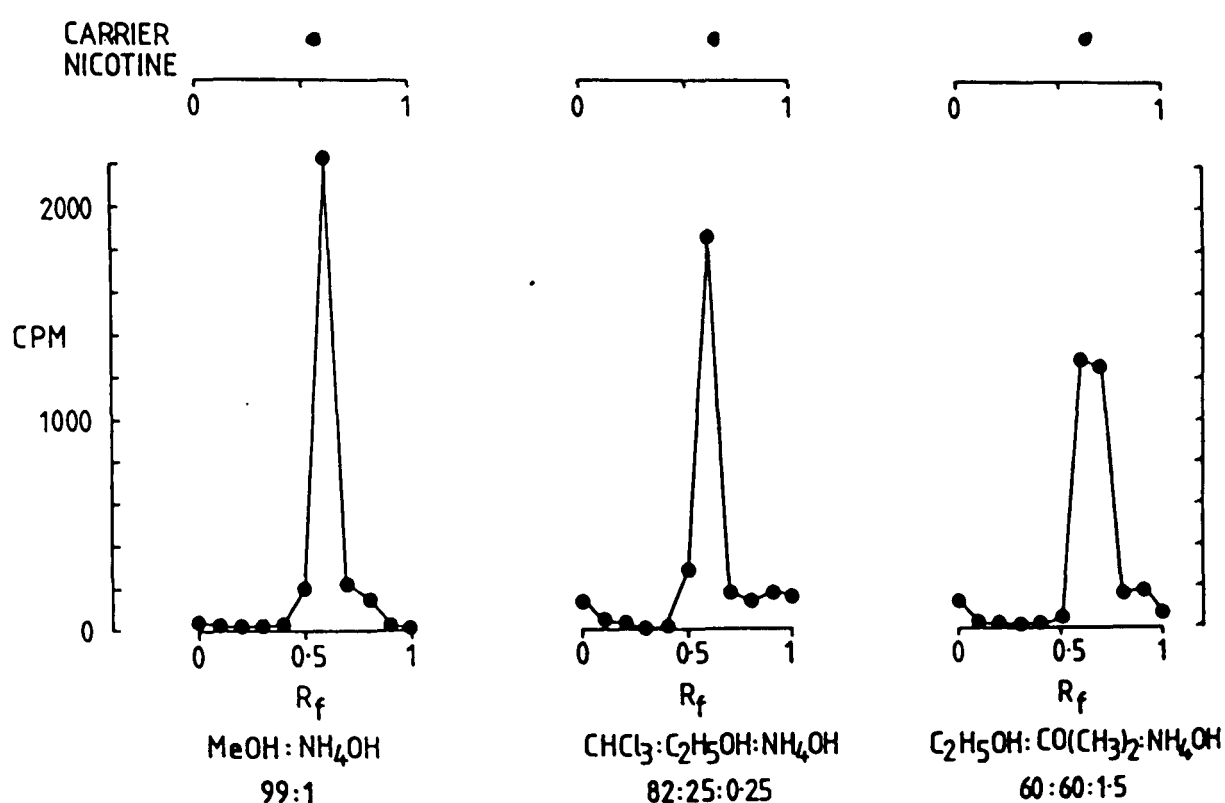
The experiments to determine the dissociation rate constant also showed that the filtration assay was an appropriate technique by which to measure the binding of  $^3\text{H}(-)$ nicotine to an olfactory membrane preparation. A typical filtration and wash of one incubation would take 5 seconds at most. Other results (figure 18) also demonstrated that the filter wash procedure was optimal for the experiment, and that up to 5x2ml washes did not result in loss of specifically bound  $^3\text{H}(-)$ nicotine from the preparation. The amount of  $^3\text{H}(-)$ nicotine which bound "specifically" to the filters themselves was negligible and was ignored in the calculations.

#### Identification of bound compound

The slight loss of bound  $^3\text{H}(-)$ nicotine versus time (figure 23) suggested either that the ligand was being degraded, that the binding sites were becoming inactive or that another change was taking place. This loss of binding with time has been seen previously in experiments on

$^3\text{H}$ (-)-nicotine binding to brain (Marks & Collins, 1982; Martin & Aceto, 1981). Bound  $^3\text{H}$ (-)-nicotine was extracted from the filters as described in materials and methods and analysed by thin layer chromatography on silica plates using three solvent systems (methanol:ammonium hydroxide, 99:1 / chloroform:ethanol:ammonium hydroxide, 82:25:0.25 / ethanol:acetone:ammonium hydroxide, 60:60:1.5).

Figure 26. Identification of Bound Compound by t.l.c.



Unlabelled nicotine was visualised under U.V. light, after which the plates were cut into 1 cm strips and placed into 10 ml of scintillant for determination of radioactivity. In control strips, this procedure recovered >90% of the radioactivity estimated to have been applied to the t.l.c. plate prior to development in the solvent.

The extracted bound  $^3\text{H}$ -compound gave a single peak of radioactivity which co-migrated with authentic nicotine (figure 26). Measurement of

radioactivity on other filters in the same experiment showed that 83 % of the binding was specific. The non-specific component was not investigated in these experiments. The extraction procedure recovered 95% of the radioactivity that was estimated to be bound to the preparation and retained on the filters, but only 75% of this was recovered in the organic fraction. The radioactivity remaining in the aqueous fraction is most likely to be associated with the included organic solvent (the aqueous fraction had the odour of ethyl acetate) but it is possible that a fraction of the radioactivity could be the N'-oxide derivative of nicotine (see appendix A) which is more water soluble than nicotine. This possibility was not considered further although there are methods to chemically derivatise and measure this metabolite (Jacob *et al.*, 1986).

The results of this experiment suggested that it was nicotine which was bound to the olfactory membranes and that there was no significant degradation of the ligand during a typical incubation. The reason for the loss of bound ligand with time (e.g. figure 23) is unclear and may be trivial, in other experiments (not shown) this effect was less noticeable.

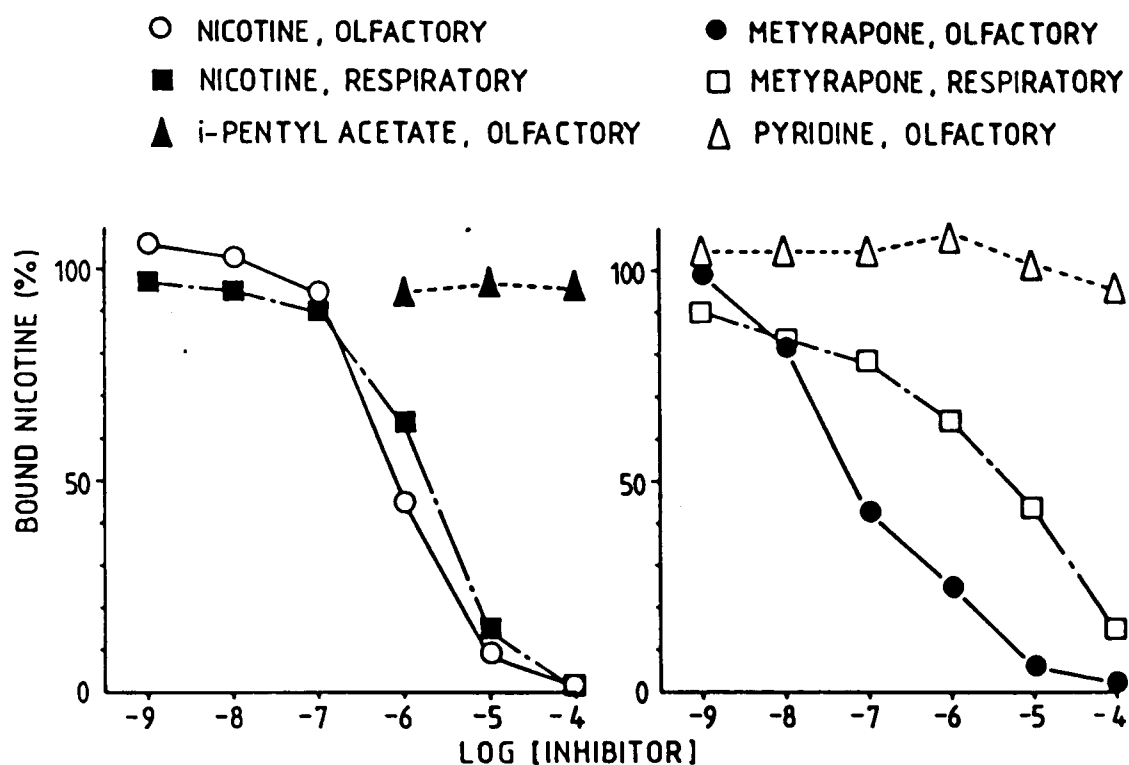
#### **Displacement studies on $^3\text{H}$ (-)-nicotine binding**

Displacement curves for  $^3\text{H}$ (-)-nicotine binding to olfactory membranes and to a similar preparation of respiratory membranes are shown in figure 27 for a selection of inhibitors added to the assay tube prior to incubation. Neither the odorant i-pentyl acetate nor the 'parent' compound and odorant pyridine, at up to mM concentrations (not illustrated) affected the binding of  $^3\text{H}$ (-)-nicotine to olfactory membranes. Nicotine and



metyrapone displaced  $^3\text{H}(-)$ nicotine from its binding site in both olfactory and respiratory membrane preparations but in a different manner from each tissue type. The Hill coefficients (not shown) of the displacement of the  $^3\text{H}(-)$ nicotine by metyrapone from both olfactory and respiratory membranes and by nicotine from olfactory membranes suggested that there may be binding site heterogeneity.

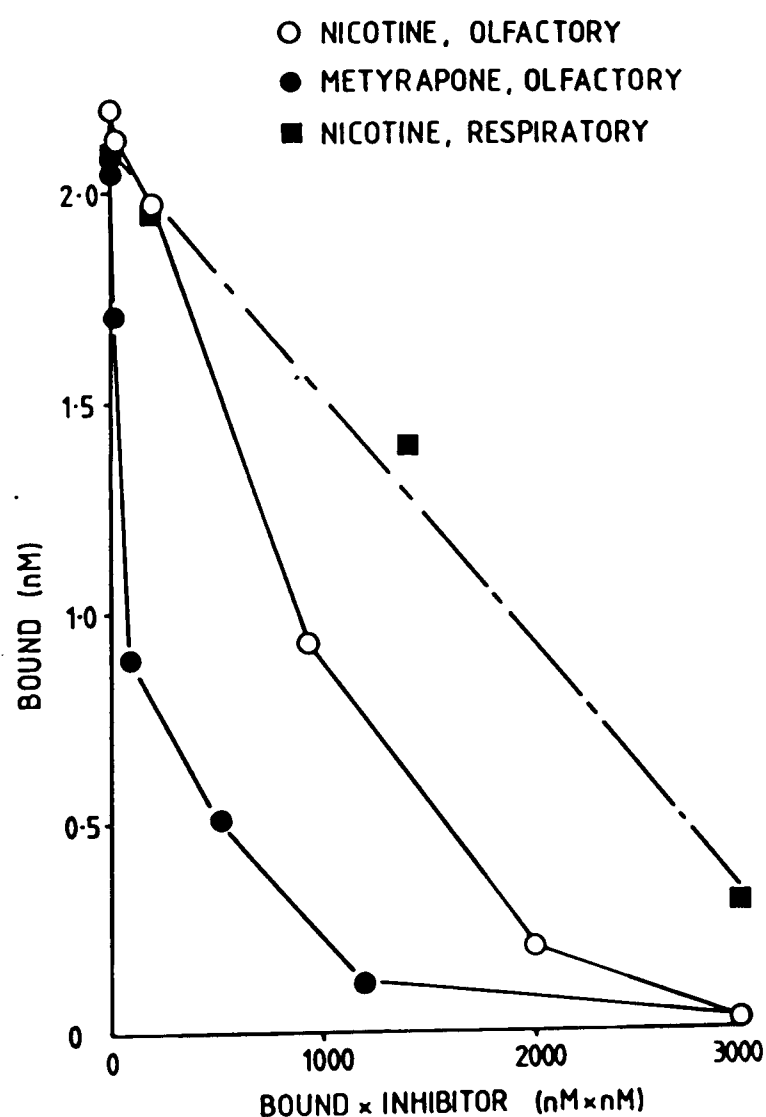
Figure 27. Displacement Curves



The Inhibitors were dissolved in the assay buffer and added to the incubation tube together with the  $^3\text{H}(-)$ nicotine, any unlabelled nicotine and buffer. The incubation was started by the addition of membranes to a final volume of 200  $\mu\text{l}$ . By using staggered additions of membranes, each tube was incubated at room temperature (typically 20°C) for the same period of time. The points show mean values ( $n=2$ ) from single typical experiments. (duplicates within 10%)

An analytical method to show this property from displacement study data has recently been described by Bylund (1986). This analysis was applied to these results and for comparison, to results which did not exhibit this apparent heterogeneity. Three typical examples of this analysis are shown in figure 28

Figure 28. Bylund Plots



Data was calculated from the values shown in figure 27 according to the following equation;  $B = - (B \times I)(1/IC_{50}) + B_0$ , where  $B$  = bound,  $I$  = Inhibitor,  $B_0$  = bound in the absence of Inhibitor (can be cross checked with experimental values). A plot of  $B$  vs  $B \times I$  gives a line (for a single site) of slope  $- 1/IC_{50}$ .

Displacement of  $^3\text{H}(-)$ nicotine from respiratory membranes by nicotine appeared to be from a single site (giving a straight line in this analysis), whereas displacement of the labelled nicotine from olfactory membranes by metyrapone appeared to be from two sites. The result for nicotine on olfactory membranes is intermediate but suggests that more than one binding site may be involved.

Graphical estimates of the  $\text{IC}_{50}$  values following Bylund plot analysis were made for comparison to those obtained by Hill plot analysis of the data. A summary of the  $\text{IC}_{50}$  values for a variety of inhibitors (many of which will be odorants in addition to being pharmacologically active) is given in table 9. Both Hill plot and Bylund plot estimates are shown.

Table 9.  $\text{IC}_{50}$  values from displacement studies on  $^3\text{H}(-)$ nicotine binding to nasal epithelium

Determinations were made at  $20^\circ\text{C}$  unless stated otherwise using  $^3\text{H}(-)$ nicotine at 19 nM final concentration, as described in the Methods section. Membranes were pre-incubated for 1 hour with  $\alpha$ -bungarotoxin before addition of the  $^3\text{H}(-)$ nicotine in order to determine the  $\text{IC}_{50}$  value for the toxin. Results are shown as; mean and standard deviation for 3 independent determinations, mean for 2 independent determinations, and are marked \* for single experiment results. A=Hill plot, B=Bylund plot, O=Olfactory, R=Respiratory, -T = bitartrate salt

Abbreviations and common names of inhibitors/odorants: DMPP (1,1-dimethyl-4-phenylpiperazinium iodide); i-pentyl acetate (i-amyl acetate); i-pentanoic acid (i-valeric acid); IBMP, (2-isobutyl-3-methoxypyrazine)

Table 9.

Inhibitor	Tissue	IC <sub>50</sub> (10 <sup>-7</sup> M) A	IC <sub>50</sub> (10 <sup>-7</sup> M) B
(-)-nicotine	O	7.1 ± 0.6	(1) 7.8 ± 2.0 (2) 75.9 ± 29.4
(-)-nicotine	R	15.1 ± 1.6	17.9 ± 1.8
(-)-nicotine 0°C	O	44.1	43.9
(-)-nicotine-T	O	12.5	13.2
(+)-nicotine-T	O	6.4	8.8
metyrapone	O	1.0 ± 0.6	(1) 0.7 ± 0.1 (2) 171.0 ± 62.0
metyrapone	R	35.4	(1) 7.6 (2) 342.0
metyrapone 0°C	O *	1.3	7.8
IBMP	O	40.7	59.4
IBMP	R *	25.7	41.4
aniline	O	1590.0	

IC<sub>50</sub> values (Hill plot analysis only) of compounds with little effect on the binding of <sup>3</sup>H(-)-nicotine to olfactory membranes;

α-bungarotoxin > 10<sup>-6</sup>M; i-pentyl acetate, pyridine, 2-pyrrolidinone, pempidine, dihydro-β-erythroidine, > 10<sup>-3</sup>M; DMPP, decamethonium, mecamlamine, atropine, i-pentanoic acid, >> 10<sup>-3</sup>M

The  $IC_{50}$  is related to the dissociation constant for the inhibitor,  $K_I$ , by the equation shown below (Cheng & Prussof, 1973).

$$K_I = \frac{IC_{50}}{1 + L / K_D}$$

where  $L$  is the concentration of radiolabelled ligand used in the displacement study and  $K_D$  is the experimentally determined dissociation constant for the labelled ligand.

In the displacement studies  $L = 19$  nM and  $K_D = 695$  nM (mean value from the two experiments at pH 8.4), giving a relationship of  $K_I = IC_{50} / 1.027$ .

The results in table 9 show that temperature affects the displacement of  $^3H(-)$ nicotine from olfactory membranes. For both nicotine and metyrapone, incubation on ice caused the apparent loss of one of the sites to which  $^3H(-)$ nicotine was bound. This temperature effect on the binding sites is also suggested from the dissociation rate constant results shown in figure 25. If this is so, then there is an accompanying shift in  $IC_{50}$  for nicotine and metyrapone displacement of  $^3H(-)$ nicotine binding.

**7-Ethoxycoumarin de-ethylase activity**

The sonication procedure caused the release of 7-ethoxycoumarin de-ethylase activity from the olfactory epithelium, even after the shortest sonication time tested (3 seconds). 7-Ethoxycoumarin de-ethylase activities of 0.43, 0.14 and 0.59 nmol/min/mg protein were measured in rat olfactory 1000×g supernatant (three separate experiments). These values represent 18%, 14% and 26% of the maximum enzyme activity recovered from the tissue following additional sonication steps (each in a change of buffer) and finally, homogenisation. This data is summarised in table 11. Also shown is the amount of  $^3\text{H}$ (-)-nicotine bound to the same fractions.

Table 10. Sonication and the release of  $^3\text{H}$ (-)-nicotine binding activity and 7-ethoxycoumarin de-ethylase activity from olfactory turbinates.

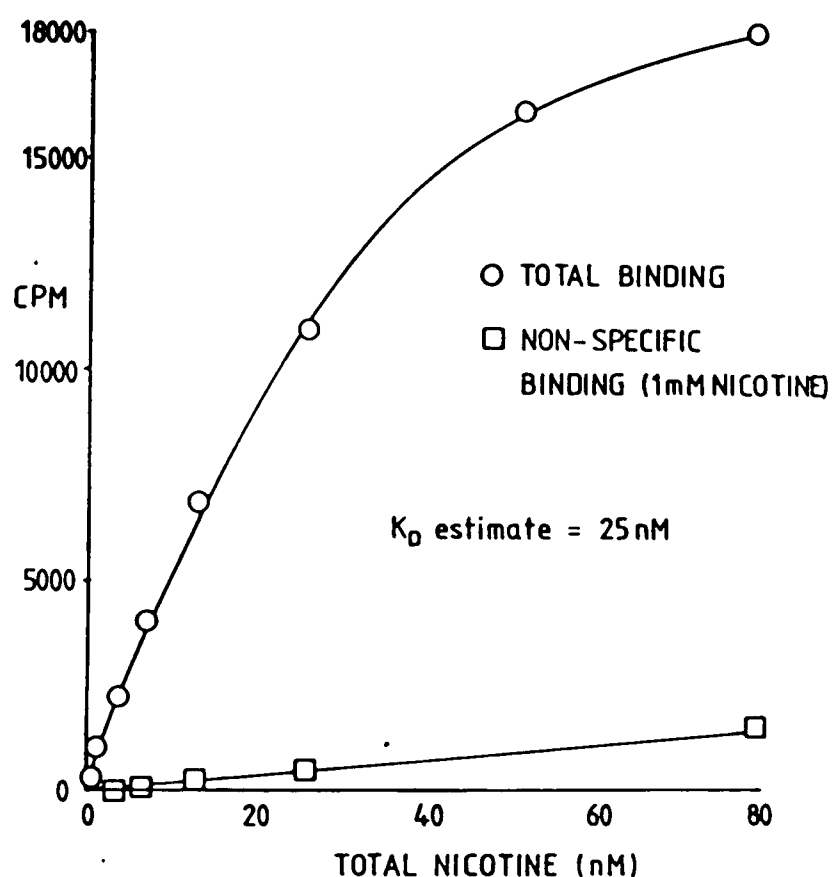
sonication	7-ethoxycoumarin de-ethylase			bound $^3\text{H}$ (-)-nicotine		
time (sec)	nmol / min / fraction			fmol / fraction		
experiment	1	2	3	1	2	3
3	0.845	0.177	1.109	318	105	671
+5	1.078	0.298	0.946	314	193	674
+12	1.831	0.048	0.530	360	43	428
+homogenisation	1.132	0.706	1.748	137	403	1658

(mean values of duplicate determinations for enzyme activity were within 15% of each other. Mean values of triplicate determinations for binding were within 10% of each other).

Rat hepatic and olfactory 7-ethoxycoumarin de-ethylase activities have been measured by other workers. These are; liver  $0.76 \pm 0.16$  ( $n=3$ ) nmol/min/mg, olfactory  $3.40 \pm 1.66$  (3) nmol/min/mg (Reed *et al.*, 1986) and liver  $0.66 \pm 0.19$  (6) nmol/min/mg, olfactory  $3.77 \pm 0.62$  (6) nmol/min/mg (Jenner & Dodd, 1988). These values are from microsomal preparations. Reed *et al.* (1986) also measured the levels of cytochrome P-450 in their preparations. These values were  $0.53 \pm 0.06$  (3) nmol/mg for hepatic microsomes and  $0.19 \pm 0.06$  (3) nmol/mg for olfactory microsomes. Hadley & Dahl (1982) give a value for cytochrome P-450 of  $0.11 \pm 0.01$  (3) nmol/mg in their olfactory microsome preparation. In all cases microsomes were prepared following homogenisation of rat olfactory turbinates.

#### Binding of $^3\text{H}(-)$ nicotine to liver microsomes

The binding of  $^3\text{H}(-)$ nicotine to hepatic microsomes (prepared by Dr. J. Jenner; one experiment) was studied for comparison with the results described by Abood *et al.* (1985) for intact hepatocytes [ $K_D=0.2$  nM,  $B_{max}=5$  fmol/mg protein] and with the results for olfactory tissue obtained here (figure 22). The direct plot of the data (figure 29) shows that specific binding to liver microsomes was measured. Scatchard analysis of the data showed a single binding site of  $K_D=25$  nM and  $B_{max}=1.28$  pmol/mg protein. These results suggest that the binding site for nicotine in olfactory membranes may be different from the binding site in liver microsomes.

Figure 29.  $^3\text{H}$ (-)-Nicotine Binding to Rat Liver Microsomes

Binding was measured in 20 mM HEPES / 0.9% NaCl buffer pH 8.4 and at 20°C. Incubations were for 15 minutes, after which the solution was filtered through 6F/C Filters (presoaked in 0.3% PEI) and washed with 4 x 3ml of ice cold buffer. The data points represent mean values (within 10%) from a single experiment.

#### Activation of olfactory adenylate cyclase by nicotine and metyrapone

The results of single experiments (performed by Dr. S.G. Shirley & Dr. C. J. Robinson) to test whether nicotine or metyrapone increased olfactory adenylate cyclase activity *in vitro* are shown in table 11.



Table 11. Activation of adenylate cyclase activity by nicotine and metyrapone.

compound	% activation above		activation as a % of	
	basal (no added odour)		1 mM acetophenone	
nicotine 1mM	27		21	
nicotine 10mM	37		29	
acetophenone 1mM	128		100	
acetophenone 1mM	112		100	
metyrapone 0.5mM	13		12	

An increase in olfactory adenylate cyclase activity was measured in the presence of nicotine and metyrapone, suggesting that both compounds have odorant properties in this assay (other compounds also vary in their ability to stimulate olfactory adenylate cyclase activity; Shirley *et al.*, 1986).

## Discussion

### <sup>3</sup>H(-)Nicotine as a probe for an olfactory receptor

There is good experimental evidence (Shirley *et al.*, 1986; Pace *et al.*, 1985; Sklar *et al.*, 1986) in support of the hypothesis (Dodd & Persaud, 1981) that olfactory transduction involves odorants binding to receptor proteins in olfactory cilia with subsequent modulation of enzymes and ion channels.

Previous work suggests that nicotine stimulates an olfactory receptor of the rat olfactory epithelium (chapter 1 & 2). It may be possible therefore to identify an olfactory receptor using the binding of <sup>3</sup>H(-)nicotine to olfactory membrane preparation. However, nicotine may interact in complex ways with the nasal mucosa.

### Possible binding sites for nicotine in nasal mucosa

Recent evidence suggests that nicotine is a stimulant for the trigeminal receptors in the nasal cavity (Silver & Walker, 1987), though the precise location of these nerve endings is not known. Nicotine is also known to pass through membranes of the oral mucosa (Squier, 1986) and therefore may pass from the vapour or aerosol phase (aerosols containing odorants may also stimulate the olfactory receptors, Mather & Dodd, 1988), through the mucus covering the olfactory epithelium and accumulate in the

underlying cells (as may many odorants, Getchell *et al.*, 1984). It is also possible that nicotine could reach the nasal epithelium via the bloodstream (Maruniak *et al.*, 1983). High concentrations of enzymes capable of metabolising nicotine have been found in the olfactory mucosa (Bond, 1983; Dahl & Hadley, 1983; Reed *et al.*, 1986; Jenner & Dodd, 1988), thus it is no surprise that nicotine and/or metabolites do accumulate in nasal mucosa (Brittebo & Tjalve, 1983; Waddell & Marlowe, 1976; Rowell *et al.*, 1983). In addition, nicotine may also interact with the odorant binding protein of the olfactory mucus (Pevsner *et al.*, 1986).

Binding of nicotine to an acetylcholine receptor of olfactory epithelium must also be considered. Although the presence of a muscarinic cholinergic receptor in nasal mucosa has been demonstrated (Hedlund & Shepherd, 1983), there is no known cholinergic input to the epithelium. That the  $^3\text{H}(-)$ nicotine binding to olfactory membranes is not affected by a variety of cholinergic agonists and antagonists (table 9) suggests that  $^3\text{H}(-)$ nicotine is not binding to a cholinergic site in the olfactory epithelium (see the results in chapter 1 also). The difference in the binding parameters between the binding described here and the binding of  $^3\text{H}(-)$ nicotine to brain membranes (e.g. in  $K_D$  and  $B_{max}$ , see table 2 of the General Introduction and e.g. in association and dissociation rate constants, see Lippiello *et al.*, 1987) also supports this conclusion. The parameters of non-cholinergic binding of nicotine to sites found on leucocyte membranes (Hoss *et al.*, 1986) or to hepatocyte membranes (Abood

*et al.*,1985a) are similar in some ways (eg. optimum binding is measured at a high pH) but different in others (eg. binding parameters and specificity), suggesting that the  $^3\text{H}(-)\text{nicotine}$  binding described here may be unique to nasal tissue.

#### Olfactory and respiratory $^3\text{H}(-)\text{nicotine}$ binding sites

The binding of  $^3\text{H}(-)\text{nicotine}$  to olfactory and respiratory membranes shows surprising differences between the two tissues which may be useful in interpreting the results. Statistical analysis (t-test) on the  $\text{IC}_{50}$  data from the displacement studies using nicotine as the inhibitor showed that the high affinity site of olfactory membranes was different (two fold tighter binding) from the binding site observed in respiratory tissue ( $0.001 < p < 0.002$ ). The lower affinity site of olfactory membranes (as identified in the Bylund plots, see fig. 28) and the binding site of respiratory membranes have similar  $\text{IC}_{50}$  values, though this does not mean they are the same binding site. In the displacement studies, respiratory membranes bound more  $^3\text{H}(-)\text{nicotine}$  than olfactory membranes under similar non-saturating concentrations of  $^3\text{H}(-)\text{nicotine}$ ,  $452 \pm 48$  fmol per mg protein (mean and standard deviation of 3 experiments) against  $338 \pm 86$  fmol per mg protein (8 experiments).

### Nicotine and nasal cytochrome P-450

7-Ethoxycoumarin de-ethylase activity has been associated with high levels of cytochrome P-450 in rat nasal preparations (Reed *et al.*, 1986). Metyrapone, an inhibitor of rat olfactory 7-ethoxycoumarin de-ethylase activity (Jenner & Dodd, 1988; and in this preparation:  $IC_{50}=7.8 \mu M$ , mean of two experiments), is also known to inhibit accumulation of nicotine in the bronchial epithelium of the mouse (Waddell & Marlowe, 1978). Metyrapone also displaced  $^3H(-)$ nicotine from its binding site in olfactory membranes (table 10). These results suggest that nicotine may interact with nasal cytochrome P-450. Since the levels of cytochrome P-450 were not measured in the  $1000\times g$  preparations, it is difficult to comment further on this possibility. However, both nicotine and metyrapone stimulated the adenylate cyclase preparation described by Shirley *et al.* (1986) suggesting that metyrapone has odorant properties. Metyrapone (figure 20) is a non-volatile compound which is able to stimulate olfactory adenylate cyclase activity when in solution. This property of non-volatile compounds has been observed previously (Shirley *et al.*, 1986). Thus, metyrapone may displace nicotine from an olfactory receptor and from another binding site in olfactory epithelium.

### Nicotine and the odorant-binding protein

It is possible that some of the nicotine is binding to the odorant-binding-protein, for example, 2-isobutyl-3-methoxypyrazine displaces  $^3H(-)$ nicotine from its binding site in both olfactory and respiratory

membrane preparations. However, there are many differences between the binding characteristics of 2-isobutyl-3-methoxypyrazine (Pevsner *et al.*, 1985; Pevsner *et al.*, 1986) and of nicotine to the olfactory mucosa. For example, the pyrazine binding protein is a soluble protein whereas evidence suggests that the nicotine binding site(s) is membrane associated (table 13), and 1-pentyl acetate is known to displace the pyrazine from its binding site (Pevsner *et al.*, 1986). It would be improper though to exclude the binding of  $^3\text{H}(-)$ nicotine to the odorant-binding-protein in these experiments without more conclusive evidence.

#### Is nicotine binding to an olfactory receptor?

Is there any evidence that  $^3\text{H}(-)$ nicotine is binding to an olfactory receptor? Although this work does not show conclusively that one of the binding sites is an olfactory receptor, there are some properties of the binding which can be compared with those predicted for an olfactory receptor from current knowledge.

For instance, it has been shown in electrophysiological studies (Shirley *et al.*, 1987a; chapter 2) that the binding constants of the olfactory receptors for some odorants are in the order of hundreds of nM. This compares favourably with the result for nicotine described here ( $K_D=695$  nM, mean of two determinations at optimum conditions for binding). It has also been suggested that the hydrophobicity of an odorant is important in determining the extent of interaction with an olfactory

receptor (Shirley *et al.*, 1987a; Senf *et al.*, 1980). This also appears to be important in the binding of  $^3\text{H}(-)$ nicotine to olfactory membranes observed in this study. The rapid association and dissociation of  $^3\text{H}(-)$ nicotine from its binding site in olfactory membranes is also consistent with the result expected for an olfactory receptor. In addition, the number of binding sites for nicotine ( $B_{\text{max}}=8.24$  pmol per mg of protein, determined from Scatchard analysis of the binding data assuming only one binding site) suggests that the binding site is a significant component of the total protein in olfactory epithelium but is not as abundant as the odorant-binding-protein (Pevsner *et al.*, 1985) for example, which is secreted in the mucus. The displacement study showed that the bound  $^3\text{H}(-)$ nicotine was completely displaced from its binding site(s) by nicotine, metyrapone and 2-isobutyl-3-methoxypyrazine (not shown). This general specificity is also consistent with the response expected from an olfactory receptor. Finally, although the binding of  $^3\text{H}(-)$ nicotine to nasal preparations is not localised to olfactory membranes alone, there is evidence from displacement studies on olfactory and nasal respiratory membrane preparations to suggest that there is a binding site unique to olfactory epithelium.

In some respects therefore, the binding site for nicotine in rat olfactory epithelium satisfies the criteria for an olfactory receptor. These have been summarised by Lancet (1986) and are shown below;

# Proposed Criteria for Identification of an Olfactory Receptor

- (1) Tissue Specificity
- (2) Enrichment in the cilia (vs. epithelium)
- (3) Glycosylation
- (4) Transmembrane disposition (integral membrane protein)
- (5) Correct bilayer concentration (major component)
- (6) Diversity (sequence heterogeneity)
- (7) Specific recognition by function-modulating reagents (antibodies, lectins)
- (8) Interaction with transducing proteins
- (9) Reconstitution of odorant modulation of enzymatic activities

Whether the  $^3\text{H}$ (-)-nicotine binding measured here includes binding to an "olfactory receptor" or is solely to another binding site in olfactory epithelium (a component of an odorant transport/clearance mechanism for instance) is difficult to determine on the basis of these results. The results of a binding study alone do not provide conclusive evidence for identification of an olfactory receptor. For example, isolation of an odorant-binding component and examination of its ability to respond to odorants in reconstitution experiments with components of the transduction mechanism would provide much more conclusive evidence (criterion 9). Clearly there is much more work to be done in order to fully understand the interaction of nicotine with the nasal epithelium and to finally isolate an odorant binding site which satisfies all the criteria for an olfactory receptor.



### Further experiments and comments

The whole-tissue experiments (EOGs) would be supplemented by adaptation studies using odorants which may interact with the same olfactory receptors for nicotine. Results from such studies would be useful in determining the structure-activity relationship of an olfactory receptor's odorant-binding site and would supplement displacement studies on  $^3\text{H}(-)\text{nicotine}$  binding. Further modification studies with other reagents may also be useful. Ideally, a reagent which binds covalently to the epithelium and selectively inhibits the nicotine EOG would be found.

There is still much work to be done in order to fully characterise the binding of nicotine to olfactory membranes. Some possible experiments are as follows;

(1) To investigate the effect of proteases and phospholipases on the binding.

(2) To further investigate the effect of temperature on the binding.

(3) An extensive study to separate the binding components into different fractions (and to measure 7-ethoxycoumarin de-ethylase activity and cytochrome P-450 levels in these fractions).

(4) A binding study with  $^3\text{H}(-)$ nicotine to purified odorant-binding protein (Pevsner *et al.*, 1985).

(5) To test a more extensive list of compounds (including odorants) in displacement studies (e.g. as done by Sloan *et al.*, 1985 for nicotine binding to brain).

(6) To further investigate the binding parameters of nicotine to respiratory tissue.

(7) To test other species for olfactory nicotine binding sites.

(8) To further study the effect of nicotine on the olfactory adenylate cyclase and to identify any other second messenger pathways stimulated by nicotine.

(9) To isolate and purify a nicotine-binding component (which could be tested for odorant activation in reconstitution experiments with components of the olfactory adenylate cyclase system).

(10) To further study the metabolism of nicotine by nasal enzymes.

Such experiments may help to identify effects and to suggest possible effects which nicotine may have on the olfactory epithelium and its function. These results may then prove to be of some clinical use with respect to chronic nicotine exposure during active smoking.

The effect of pharmacologically active odorants on the olfactory epithelium and on other nasal tissue should also be investigated. Such compounds may be of scientific and commercial value.



References

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## References

ABOOD, L.G., GRASSI, S. & COSTANZA, M. (1983)

Binding of Optically Pure (-)-[<sup>3</sup>H]nicotine to Rat Brain Membranes.  
FEBS Letts. 157, 147-149

ABOOD, L.G., GRASSI, S., JUNIG, J., CRANE, A. & COSTANZO, M. (1985a)

Specific Binding and Metabolism of (-)- and (+)- [<sup>3</sup>H]-Nicotine in  
Isolated Rat Hepatocytes and Hepatocyte Membranes.  
Arch. int. Pharmacodyn. 273, 62-73

ABOOD, L.G., GRASSI, S. & NOGGLE, H.D. (1985b)

Comparison of the Binding of Optically Pure (-)- and (+)-  
[<sup>3</sup>H] Nicotine to Rat Brain Membranes.  
Neurochemical Research 10, 259-267

ADAMEK, G.D., GESTELAND, R.C., MAIR, R.G. & OAKLEY, B. (1984)

Transduction Physiology of Olfactory Receptor Cilia.  
Brain Research 310, 87-97

AMOORE, J.E. (1967)

Specific Anosmia: A Clue to the Olfactory Code.  
Nature Lond. 214, 1095-1098

ANHOLT, R.R.H., MUMBY, S.M., STOFFERS, D.A., GIRARD, P.R., KUO, J.F. &  
SNYDER, S.H. (1987)

Transduction Proteins of Olfactory Receptor Cells: Identification of  
Guanine Nucleotide Binding Proteins and Protein Kinase C.  
Biochemistry 26, 788-795

ANHOLT, R.R.H., MURPHY, K.M.M., MACK, G.E. & SNYDER, S.H. (1984)

Peripheral-Type Benzodiazepine Receptors in the Central Nervous  
System: Localization to Olfactory Nerves.  
The Journal of Neuroscience 4, 593-603

BALFOUR, D.J.K. (1982)

The Pharmacology of Nicotine Dependence: A Working Hypothesis.  
Pharmac. Ther. 15, 239-250

BARLOW, R.B. & HAMILTON, J.T. (1965)

The Stereospecificity of Nicotine.  
Brit. J. Pharmacol. 25, 206-212

BARNARD, E.A. & DEMOLIOU-MASON, C. (1983)

Molecular Properties of Opioid Receptors.  
Brit. Med. Bull. 39, 37-45

BENOWITZ, N.L. (1986)

Clinical Pharmacology of Nicotine.

Ann. Rev. Med. 37, 21-32

BENOWITZ, N.L., KUYT. F., JACOB, P., JONES, R.T. & OSMAN, A-L. (1983)

Cotinine Disposition and Effects.

Clin. Pharmacol. Ther. 34, 604-611

BENWELL, M.E.M. & BALFOUR, D.J.K. (1985)

Nicotine Binding to Brain Tissue from Drug-Naive and Nicotine-Treated Rats.

J. Pharm. Pharmacol. 37, 405-409

BIRDSALL, N.J.M., HULME, E.C., STOCKTON, J., BURGEN, A.S.V., BERRIE, C.P.,

HAMMER, R., WONG, E.H.F. & ZIGMOND, M.J. (1983)

Muscarinic Receptor Subclasses: Evidence from Binding Studies,

pp. 323-336 in: CNS Receptors - from Molecular Pharmacology to

Behavior, editors Mandell, P. & DeFeudis, F.V., Raven Press, New York.

BOEYNAEMS, J.M. & DUMONT, J.E. (1980)

Outlines of Receptor Theory.

Elsevier/North-Holland Biomedical Press, Amsterdam, New York, Oxford.



BOND, J.A. (1983)

Some Biotransformation Enzymes Responsible for Polycyclic Aromatic Hydrocarbon Metabolism in Rat Nasal Turbinates: Effects on Enzyme Activities of *in vitro* Modifiers and Intraperitoneal and Inhalation Exposure of Rats to Inducing Agents.

Cancer Res. 43, 4805-4811

BOWMAN, E.R., HANSSON, E., TURNBULL, L.B., McKENNIS, H. & SCHMITTERLOW, C.G.  
(1964)

Disposition and Fate of (-)-cotinine- $H^3$  in the Mouse.

J. Pharm. Exp. Ther. 143, 301-308

BREWER, STERNLICHT, MARCUS & GROLLMAN (1973)

Binding of  $^{13}C$ -enriched  $\alpha$ -methyl-D-glucopyranoside to Concanavalin A as Studied by Carbon Magnetic Resonance.

Proc. Natl. Acad. Sci. USA 70, 1007-1011

BRITTEBO, E.B. & TJALVE, H. (1983)

Metabolism of N-Nitrosamines by the Nasal Mucosa. in: Nasal Tumors in Animals and Man, Vol.3, (Reznik, G. & Stinson, S.F., eds.) pp. 233-250, CRC Press, Florida

BRUNS, R.F., LAWSON-WENDLING, K. & PUGSLEY, T.A. (1983)

A Rapid Filtration Assay for Soluble Receptors Using  
Polyethyleneimine-Treated Filters.

Anal. Biochem. 132, 74-81

BYLUND, D.B. (1986)

Graphic Presentation and Analysis of Inhibition Data from  
Ligand-Binding Experiments.

Anal. Biochem. 159, 50-57

CATTABENI, F. & NICOSIA, S., eds. (1984)

Principles and Methods in Receptor Binding.

NATO ASI series A: Life Sciences Vol.72, Plenum.

CAVAGGIONI, A., SORBI, R.T., KEEN, J.N., PAPPIN, D.J.C. & FINDLAY, J.B.C. (1987)

Homology Between the Pyrazine-Binding Protein from Nasal Mucosa  
and Major Urinary Proteins.

FEB Letts. 212, 225-228

CHEN, Z. & LANCET, D. (1984)

Membrane Proteins Unique to Vertebrate Olfactory Cilia: Candidates  
for Sensory Receptor Molecules.

Proc. Natl. Acad. Sci. USA 81, 1859-1863

CHEM, Z., PACE, U., HELDMAN, J., SHAPIRA, A. & LANCET, D. (1986a)

Isolated Frog Olfactory Cilia: A Preparation of Dendritic Membranes  
From Chemosensory Neurons.

J. Neurosci. 6, 2146-2154

CHEM, Z., PACE, U., RONEN, D. & LANCET, D. (1986b)

Polypeptide gp95. A Unique Glycoprotein of Olfactory Cilia with  
Transmembrane Receptor Properties.

J. Biol. Chem. 261, 1299-1305

CHENG, Y.C. & PRUSSOF, W.H. (1973)

Relationship Between the Inhibition Constant  $K_i$  and the Concentration  
of Inhibitor Which Causes Fifty Percent Inhibition ( $IC_{50}$ ) of an Enzyme  
Reaction.

Biochem. Pharmacol. 22, 3099-3108

CLARKE, P.B.S., PERT, C.B. & PERT, A. (1984)

Authoradiographic Distribution of Nicotine Receptors in Rat Brain.

Brain Res. 323, 390-395

COLLINS, A.C., EVANS, C.B., MINER, L.L. & MARKS, M.J. (1986)

Mecamylamine Blockade of Nicotine Responses: Evidence for Two Brain  
Nicotinic Receptors.

Pharmacol. Biochem. Behav. 24, 1767-1773

COSTA, L.G. & MURPHY, S.D. (1983)

[<sup>3</sup>H]Nicotine Binding in Rat Brain: Alteration after Chronic  
Acetylcholinesterase Inhibition.

J. Pharm. Exp. Ther. 226, 392-397

CRIADO, M., SARIN, V., FOX, J.L. & LINDSTROM, J. (1985)

Structural Localization of the Sequence  $\alpha$  235-242 of the Nicotinic  
Acetylcholine Receptor.

Biochem. Biophys. Res. Comm. 128, 864-871

DAHL, A.R. & HADLEY, W.M. (1983)

Formaldehyde Production Promoted by Rat Nasal Cytochrome  
P-450-Dependent Monooxygenases with Nasal Decongestants, Essences,  
Solvents, Air Pollutants, Nicotine, and Cocaine as Substrates.

Tox. Appl. Pharmacol. 67, 200-205

DAHL, A.R., HADLEY, W.M., HAHN, F.F., BENSON, J.M. & McCLELLAN, R.O. (1982)

Cytochrome P-450-Dependent Mono-Oxygenases in Olfactory Epithelium of  
Dogs: Possible Role in Tumorigenicity.

Science 216, 57-59

DAVIES, B.D., HOSS, W., LIN, J-P, & LIONETTI, F. (1982)

Evidence for a Noncholinergic Nicotine Receptor on Human Phagocytic  
Leukocytes.

Mol. Cell. Biochem. 44, 23-31

DAWSON, G.W. & VESTAL, R.E. (1982)

Smoking and Drug Metabolism.

Pharmac. Ther. 15, 207-221

DAWSON, R.M. (1984)

The Affinity of [<sup>3</sup>H]Quinuclidinyl Benzilate and Some  
Other Radioligands for Glass Microfiber and Cellulose Filters in Some  
Common Liquid Scintillation Solvents.

Anal. Biochem. 139, 493-501

DAWSON, R.M.C., ELLIOTT, D.C., ELLIOTT, W.H. & JONES, K.M. (1986)

Data for Biochemical Research. Third Edition.

Clarendon Press, Oxford

DODD, G. & PERSAUD, K. (1981)

Biochemical Mechanisms in Vertebrate Primary Olfactory Neurons.

pp.333-357 in: Biochemistry of Taste and Olfaction, eds Cagan, R.H. &  
Kare, M.R. Academic Press

DODD, G.H. & SQUIRRELL, D.J. (1980)

Structure and Mechanism in the Mammalian Olfactory System.

Symp. zool. Soc. Lond. 45, 35-56

DUCHAMP, A. (1982)

Electrophysiological Responses of Olfactory Bulb Neurons to Odour Stimuli in the Frog. A Comparison with Receptor Cells.

Chem. Senses 7, 191-210

DUCHAMP, A. & SICARD, G. (1984)

Influence of Stimulus Intensity on Odour Discrimination by Olfactory Bulb Neurons as Compared with Receptor Cells.

Chem. Senses 8, 355-366

ENZELL, C.R. (1981)

Influence of Curing on the Formation of Tobacco Flavour, pp.476 in: Flavour '81, Editor Schreier, P., de Gruyter, Berlin, New York

FENTON, R.A. & DOBSON, J.G. (1985)

Nicotine Increases Heart Adenosine Release, Oxygen Consumption, and Contractility.

Am. J. Physiol. 249 (Heart Circ. Physiol. 18): H463-H469

FESEENKO, E.E., NOVOSELOV, V.I. & KRAPIVINSKAYA, L.D. (1979)

Molecular Mechanisms of Olfactory Reception. IV. Some Biochemical Characteristics of the Camphor Receptor from Rat Olfactory Epithelium.

Biochim. Biophys. Acta. 587, 424-433

FESENKO, E.E., NOVOSELOV, V.I. & NOVIKOV, J.V. (1985)

Molecular Mechanisms of Olfactory Reception. VI. Kinetic  
Characteristics of Camphor Interaction with Binding Sites of Rat  
Olfactory Epithelium.

Biochim. Biophys. Acta. 839, 268-275

FRANKENBURG, W.G. & VAITEKUNAS, A.A. (1957)

The Chemistry of Tobacco Fermentation. I. Conversion of the Alkaloids.  
D. Identification of Cotinine in Fermented Leaves.

J. Am. Chem. Soc 79, 149-151

GALPER, J.B., KLEIN, W. & CATTERALL, W.A. (1977)

Muscarinic Acetylcholine Receptors in Developing Chick Heart.

J. Biol. Chem. 252, 8692-8699

GENNINGS, J.N., GOWER, D.B. & BANNISTER, L.H. (1977)

Studies on the Receptors to 5 $\alpha$ -Androst-16-en-3-one and  
5 $\alpha$ -Androst-16-en-3 $\alpha$ -ol in Sow Nasal Mucosa.

Biochim. Biophys. Acta. 496, 547-556

GETCHELL, M.L. & GESTELAND, R.C. (1972)

The Chemistry of Olfactory Reception: Stimulus-Specific Protection  
from Sulfhydryl Reagent Inhibition.

Proc. Natl. Acad. Sci. USA 69, 1494-1498

GETCHELL, T.V. (1986)

Functional Properties of Vertebrate Olfactory Receptor Neurons.

Physiological Reviews 66, 772-818

GETCHELL, T.V., MARGOLIS, F.L. & GETCHELL, M.L. (1984)

Perireceptor and Receptor Events in Vertebrate Olfaction.

Prog. Neurobiol. 23, 317-345

GRANT, C.W.M. & PETERS, M.W. (1984)

Lectin-Membrane Interactions. Information from Model Systems.

Biochim. Biophys. Acta 779, 403-422

GUNTHER, G.R., WANG, J.L., YAHARA, I., CUNNINGHAM, B.A. & EDELMAN, G.M. (1973)

Concanavalin A Derivatives with Altered Biological Activities.

Proc. Natl. Acad. Sci. USA 70, 1012-1016

HADLEY, W.M. & DAHL, A.R. (1982)

Cytochrome P-450-Dependent Monooxygenase Activity in Rat Nasal  
Epithelial Membranes.

Toxicol. Lett. 10, 417-422

HALL, G.H. (1970)

Effects of Nicotine and Tobacco Smoke on the Electrical Activity of  
the Cerebral Cortex and Olfactory Bulb.

Br. J. Pharmac. 38, 271-286



HALL, G.H. (1982)

Pharmacological Responses to the Intracerebral Administration of Nicotine.

Pharmac. Ther. 15, 223-238

HAMILTON, S.L., PRATT, D.R. & EATON, D.C. (1985)

Arrangement of the Subunits of the Nicotinic Acetylcholine Receptor of *Torpedo californica* as Determined by  $\alpha$ -Neurotoxin Cross-Linking.

Biochemistry 24, 2210-2219

HARTREE, E.F. (1972)

Determination of Protein: A Modification of the Lowry Method that gives a Linear Photometric Response.

Anal. Biochem. 48, 422-427

HEDLUND, B. & SHEPHERD, G.M. (1983)

Biochemical Studies on Muscarinic Receptors in the Salamander Olfactory Epithelium.

FEBS Lett. 162, 428-431

HELDMAN, J. & LANCET, D. (1986)

Cyclic AMP-Dependent Protein Phosphorylation in Chemosensory Neurons: Identification of Cyclic Nucleotide-Regulated Phosphoproteins in Olfactory Cilia.

J. Neurochem. 47, 1527-1533

HEWNINGFIELD, J.E., MIYASATO, K. & JASINSKI, D.R. (1985)

Abuse Liability and Pharmacodynamic Characteristics of Intravenous  
and Inhaled Nicotine.

J. Pharmacol. Exp. Ther. 234, 1-12

HOFFMANN, D., LAVOIE, E.J. & HECHT, S.S. (1985)

Nicotine: A Precursor for Carcinogens.

Cancer Letters 26, 67-75

HOKIN, L.E. (1985)

Receptors and Phosphoinositide Generated Second Messengers.

Ann. Rev. Biochem. 54, 205-235

HOSS, W., LIN, J-P. F, MATCHET, S. & DAVIES, B.D. (1986)

Characterization of Noncholinergic Nicotine Receptors on Human  
Granulocytes.

Biochem. Pharmacol. 35, 2367-2372

HUNT, L.A. & KELLEY, K.S. (1984)

Nicotine-Stimulated Proteins in Mouse Cells are Distinct from  
Heat-Shock Proteins.

Biochem. J. 224, 87-92

HUQUE, T. & BRUCH, R.C. (1986)

Odorant - and Guanine Nucleotide - Stimulated Phosphoinositide  
Turnover in Olfactory Cilia.

Biochem. Biophys. Res. Comm. 137, 36-43

IRVINE, R.F. (1987)

The Metabolism and Function of Inositol Phosphates.

Biochem. Soc. Trans. 15, 122-123

JACKSON, K.E. (1941)

Alkaloids of Tobacco.

Chem. Rev. 29, 123-197

JACOB, P., BENOWITZ, N.L., YU, L. & SHULGIN, A.T. (1986)

Determination of Nicotine N-Oxide by Gas Chromatography following  
Thermal Conversion to 2-Methyl-6-(3-pyridyl)tetrahydro-1,2-oxazine.

Anal. Chem. 58, 2218-2221

JENNER, J & DODD, G.H. (1988)

The Interaction of Metyrapone and  $\alpha$ -Naphthoflavone with Rat  
Olfactory Cytochrome P-450.

Biochem. Pharmacol. in press

KAUER, J.S. & MOULTON, D.G. (1974)

Responses of Olfactory Bulb Neurones to Odour Stimulation of Small Nasal Areas in the Salamander.

J. Physiol. 243, 717-737

KEMP, G. & MORLEY, B.J. (1986)

Ganglionic nAChRs and High-Afinity Nicotinic Binding Sites are not Equivalent.

FEBS Lett. 205, 265-268

KEVERNE, E.B., MURPHY, C.L., SILVER, W.L., WYSOCKI, C.J. & MEREDITH, M. (1986)

Non-olfactory Chemoreceptors of the Nose: Recent Advances in Understanding the Vomeronasal and Trigeminal Systems.

Chem. Senses 11, 119-113

KURIHARA, K. & KOYAMA, K. (1972)

High Activity of Adenylate Cyclase in Olfactory and Gustatory Organs.

Biophys. Res. Comm. 48, 30-34

LABARCA, P., SIMON, S.A. & ANHOLT, R.R.H. (1987)

Activation of Odorants of a Multistate Cation Channel from Olfactory Cilia.

PNAS. USA. submitted.

LANCET, D. (1986)

Vertebrate Olfactory Reception.

Ann. Rev. Neurosci. 9, 329-356

LANCET, D., GREER, C.A., KAUER, J.S. & SHEPHERD, G.M. (1982)

Mapping of Odor-Related Neuronal Activity in the Olfactory Bulb by  
High-Resolution 2-deoxyglucose Autoradiography.

Proc. Natl. Acad. Sci. USA 79, 670-674

LEE, K-H., WELLS, R.G. & REED, R.R. (1987)

Isolation of an Olfactory cDNA: Similarity to Retinol-Binding Protein  
Suggests a Role in Olfaction.

Science 235, 1053-1056

LEGLER, G., MULLER-PLATZ, C.M., MENTGES-HETTKAMP, M., PFLIEGER, G. &  
JULICH, E. (1985)

On the Chemical Basis of the Lowry Protein Determination.

Anal. Biochem. 150, 278-287

LEVITZKI, A. (1984)

Chapter 1, Techniques of Ligand Binding to Receptors, pp.7-18

Chapter 2, Analysis of Binding Data, pp.19-36, In: Receptors. A  
Quantitative Approach.

Benjamin/Cummings Publishing Company, Inc., California, London.

LINDBERG, S., HYBBINETTE, J-C. & MERCKE, U. (1985)

Effects of Nicotine Bitartrate on Mucociliary Activity.

Eur. J. Respir. Dis. 66, 40-46

LIPPIELLO, P.M. & FERNANDES, K.G. (1986)

The Binding of L-[<sup>3</sup>H]Nicotine to a Single Class of High Affinity Sites  
in Rat Brain.

Mol. Pharmacol. 29, 448-454

LIPPIELLO, P.M., SEARS, S.B. & FERNANDES, K.G. (1987)

Kinetics and Mechanism of L-[<sup>3</sup>H]Nicotine Binding to Putative High  
Affinity Receptor Sites in Rat Brain.

Mol. Pharmacol. 31, 392-400

MACKAY-SIM, A. & KUBIE, J.L. (1981)

The Salamander Nose: A Model System for the Study of Spacial Coding  
of Olfactory Quality.

Chem. Senses 6, 249-257

MAELICKE, A. (1987)

Allosteric Interactions Between Distinct Structural Domains at the  
Nicotinic Acetylcholine Receptor.

Biochem. Soc. Trans 15, 108-112

MALIWAL, B.P. & GUTHRIE, F.E. (1981a)

Interaction of Insecticides with Human Plasma Lipoproteins.

Chem. Biol. Interactions 35, 177-188

MALIVAL, B.P. & GUTHRIE, F.E. (1981*b*)

Interaction of Insecticides with Human Serum Albumin.

Mol. Pharmacol. 20, 138-144

MANGAN, G.L. & GOLDING, J.F. (1984)

The Psychopharmacology of Smoking

Cambridge University Press

MARKS, M.J. & COLLINS, A.C. (1982)

Characterization of Nicotine Binding in Mouse Brain and Comparison  
with the Binding of  $\alpha$ -bungarotoxin and Quinuclidinyl Benzilate.

Mol. Pharmacol. 22, 554-564

MARKS, M.J., ROMM, E., BEALER, S.M. & COLLINS, A.C. (1985*a*)

A Test Battery for Measuring Nicotine Effects in Mice.

Pharmacol. Biochem. Behav. 23, 325-330

MARKS, M.J., STITZEL, J.A. & COLLINS, A.C. (1985*b*)

Time Course Study of the Effects of Chronic Nicotine Infusion on  
Drug Response and Brain Receptors.

J. Pharmacol. Exp. Ther. 235, 619-628

MARKS, M.J., STITZEL, J.A., ROMM, E., VEHNER, J.M. & COLLINS, A.C. (1986)

Nicotinic Binding Sites in Rat and Mouse Brain: Comparison of  
Acetylcholine, Nicotine, and  $\alpha$ -Bungarotoxin.

Mol. Pharmacol. 30, 427-436

MARTIN, B.R. & ACETO, M.D. (1981)

Nicotine Binding Sites and Their Localization in the  
Central Nervous System.

Neurosci. Biobehav. Rev. 5, 473-478

MARUNIAK, J.A., SILVER, W.L. & MOULTON, D.G. (1983)

Olfactory Receptors Respond to Blood-Borne Odorants.

Brain Research 265, 312-316

MASON, J.R., CLARK, L. & MORTON, T.H. (1984)

Selective Deficits in the Sense of Smell Caused by Chemical  
Modification of the Olfactory Epithelium.

Science 226, 1092-1094

MASON, J.R. & MORTON, T.H. (1984)

Fast and Loose Covalent Binding of Ketones as a Molecular Mechanism  
in Vertebrate Olfactory Receptors.

Tetrahedron 40, 483-492



MATHER, R.A. & DODD, G.H. (1988)

The Delivery of an Aqueous Aerosol Containing Non-Volatile Odorants  
to the Olfactory Epithelium of the Rat.

Chem. Senses in press

McCOY, G.D., HOWARD, P.C. & DeMARCO, G.J. (1986)

Characterization of Hamster Liver Nicotine Metabolism. I. Relative  
Rates of Microsomal C and N Oxidation.

Biochem. Pharmacol. 35, 2767-2773

McKENZIE, G.H., SAWYER, & NICHOL (1972)

The Molecular Weight and Stability of Concanavalin A.

Biochim. Biophys. Acta 263, 283-293

MENCO, B.Ph.M. (1980)

Qualitative and Quantitative Freeze-Fracture Studies on Olfactory and  
Nasal Respiratory Epithelial Surfaces of Frog, Ox, Rat and Dog.

IA. General Survey.

Cell Tissue Res. 207, 183-209

MENCO, B.Ph.M. (1983)

The Ultrastructure of Olfactory and Nasal Respiratory Epithelium  
Surfaces. pp. 45-102 in: Nasal Tumors in Animals and Man. Vol.1 eds  
Reznik, G. & Stinson, S.F., CRC press Florida

MENEVSE, A., DODD, G. & POYNTER, T.M. (1977)

Evidence for the Specific Involvement of Cyclic AMP in the Olfactory Transduction Mechanism.

Biochem. Biophys. Res. Comm. 77, 671-677

MENEVSE, A., DODD, G. & POYNTER, T.M. (1978)

A Chemical-Modification Approach to the Olfactory Code. Studies with a Thiol-Specific Reagent.

Biochem. J. 176, 845-854

MESSING, A., BIZZINI, B. & GONATAS, N.K. (1984)

Concanavalin A Inhibits Nicotinic Acetylcholine Receptor Function in Cultured Chick Ciliary Ganglion Neurons.

Brain Res. 303, 241-249

MICHELL, R.H. & KIRK, C. (1986)

G-Protein Control of Inositol Phosphate Hydrolysis.

Nature (London) 323, 112-113

MOZELL, M.M. & JAGODOWICZ, M. (1973)

Chromatographic Separation of Odorants by the Nose: Retention Times Measured Across *in vivo* Olfactory Mucosa.

Science 181, 1247-1249

MUSTAPARTA, H. (1971)

Spatial Distribution of Receptor-Responses to Stimulation with  
Different Odours.

Acta. physiol. scand. 82, 154-166

NAKAMURA, T. & GOLD, G.H. (1987)

A Cyclic Nucleotide-Gated Conductance in Olfactory Receptor Cilia.  
Nature Lond. 325, 442-444

NAKAYAMA, H., NAKASHIMA, T. & KUROGOCHI, Y. (1985)

Cytochrome P-450-Dependent Nicotine Oxidation by Liver Microsomes  
of Guinea Pigs. Immunochemical Evidence with Antibody against  
Phenobarbital-Inducible Cytochrome P-450.

Biochem. Pharmacol. 34, 2281-2286

OTTOSON, D. (1970)

Electrical Signs of Olfactory Transducer Action, pp. 343-354, in:  
Taste and Smell in Vertebrates, eds. Wolstenholme, G.E.W. & Knight, J.  
J & A Churchill, London

PACE, U., HANSKI, E., SALOMON, Y. & LANCET, D. (1985)

Odorant-Sensitive Adenylate Cyclase May Mediate Olfactory Reception.  
Nature Lond. 316, 255-258

PACE, U. & LANCET, D. (1986)

Olfactory GTP-Binding Protein: Signal Transducing Polypeptide of  
Vertebrate Chemosensory Neurons.

Proc. Natl. Acad. Sci. USA 83, 4947-4951

PEDERSEN, P.E., JASTREBOFF, P.J., STEWART, W.B. & SHEPHERD, G.M. (1986)

Mapping of an Olfactory Receptor Population that Projects to a  
Specific Region in the Rat Olfactory Bulb.

J. Comp. Neurol. 250, 93-108

PELOSI, P., BALDACCINI, N.E. & PISANELLI, A.M. (1982)

Identification of a Specific Olfactory Receptor for  
2-isobutyl-3-methoxypyrazine.

Biochem. J. 201, 245-248

PERSAUD, K.C., WOOD, P.H., SQUIRRELL, D.J. & DODD, G.H. (1981)

Biochemical Studies in Olfaction.

Biochem. Soc. Trans. 9, 107-108

PEVSNER, J., TRIFILETTI, R.R., STRITTMATTER, S.M. & SNYDER, S.H. (1985)

Isolation and Characterization of an Olfactory Receptor Protein for  
Odorant Pyrazines.

Proc. Natl. Acad. Sci. USA. 82, 3050-3054

PEVSNER, J., SKLAR, P.B. & SNYDER, S.H. (1986)

Odorant-Binding Protein: Localization to Nasal Glands and Secretions.  
Proc. Natl. Acad. Sci. USA. 83, 4942-4946

PILOTTI, A. & ENZELL, C.R. (1976)

Studies on the Identification of Tobacco Alkaloids, Their Mammalian  
Metabolites and Related Compounds by Gas Chromatography - Mass  
Spectrometry.  
Beitrage zur Tabakforschung 8, 339-349

POLAK, E.H. (1973)

Multiple Profile-Multiple Receptor Site Model for Vertebrate Olfaction.  
J. Theor. Biol. 40, 469-484

PRICE, S. (1977)

Specific Anosmia to Geraniol in Mice.  
Neurosci. Letts. 4, 49-50

PRICE, S. (1978)

Anisole Binding Protein from Dog Olfactory Epithelium.  
Chem. Sens. Flav. 3, 51-55

RHEIN, L.D. & CAGAN, R.H. (1980)

Biochemical Studies of Olfaction: Isolation, Characterization, and Odorant Binding Activity of Cilia from Rainbow Trout Olfactory Rosettes.

Proc. Natl. Acad. Sci. USA. **77**, 4412-4416

REED, C.J., LOCKE, E.A. & DE MATTEIS, F. (1986)

NADPH:cytochrome P-450 Reductase in Olfactory Epithelium. Relevance to Cytochrome P-450-dependent Reactions.

Biochem. J. **240**, 585-592

REVIAL, M.F., SICARD, G., DUCHAMP, A. & HOLLEY, A. (1982a)

New Studies on Odour Discrimination in the Frog's Olfactory Receptor Cells. I. Experimental Results.

Chem. Senses **7**, 175-190

REVIAL, M.F., SICARD, G., DUCHAMP, A. & HOLLEY, A. (1982b)

New Studies on Odour Discrimination in the Frog's Olfactory Receptor Cells. II. Mathematical Analysis of Electrophysiological Responses.

Chem. Senses **8**, 179-194

ROGERS, K.E., GRILLO, M., SYDOR, W., POONIAN, M. & MARGOLIS, F.L. (1985)

Olfactory Neuron-Specific Protein is Translated from a Large Poly(A)<sup>+</sup> mRNA.

Proc. Natl. Acad. Sci. USA **82**, 5218-5222

ROMANO, C. & GOLDSTEIN., A. (1980)

Stereoespecific Nicotine Receptors on Rat Brain Membranes.

Science 210, 647-650

ROSCOE, J.T. (1975)

Fundamental Research Statistics for the Behavioral Sciences, 2nd

Edition Holt, Rineherd & Winston Inc.

ROWELL, P.P., HURST, H.E., MARLOWE, C. & BENNETT, B.D. (1983)

Oral Administration of Nicotine: Its Uptake and Distribution After  
Chronic Administration to Mice.

J. Pharm. Meths. 9, 249-261

SASAGAWA, S., SUZUKI, K., SAKATANI, T. & FUJIKURA, T. (1985)

Effects of Nicotine on the Functions of Human Polymorphonuclear  
Leukocytes *in vitro*.

J. Leukocyte Biol. 37, 493-502

SEEMAN, J.I. (1984)

Recent Studies in Nicotine Chemistry. Conformational Analysis,  
Chemical Reactivity Studies, and Theoretical Modeling.

Heterocycles 22, 165-193

SEEMAN, J.I., SECOR, H.V., HOWE, C.R., CHAVDARIAN, C.G. & MORGAN, L.W. (1983)

Organometallic Methylation of Nicotine and Nicotine N-Oxide. Reaction  
Pathways and Racemization Mechanisms.

J. Org. Chem. 48, 4899-4904

SEEMAN, J.I., CHAUDARIAN, C.G., KORNFIELD, R.A. & NAWORAL, J.D. (1985)

Nicotine Chemistry. The Addition of Organolithium Reagents  
to (-)-Nicotine

Tetrahedron 41, 595-602

SENF, W., MENCÓ, B.Ph.M., PINTER, P.H. & DUYVESTYEN, P. (1980)

Determination of Odor Affinities Based on the Dose-Response  
Relationship of the Frog's Electro-Olfactogram.

Experientia 36, 213-214

SERSHEN, H. & LAJTHA, A. (1979)

The Effect of Nicotine on the Metabolism of Brain Proteins.

Neuropharmacology 18, 763-766

SERSHEN, H., REITH, M.E.A., BANAY-SCHWARTZ, M. & LAJTHA, A. (1982)

Effects of Prenatal Administration of Nicotine on Amino Acid Pools,  
Protein Metabolism, and Nicotine Binding in the Brain.

Neurochem. Res. 7, 1515-1522

SERSHEN, H., REITH, M.E.A., LAJTHA, A. & GENNARO Jr, J. (1981)

Noncholinergic, Saturable Binding of (±)-[<sup>3</sup>H]Nicotine to Mouse Brain.

J. Receptor Res. 2, 1-15



SHARIF, N.A. & BURT, D.R. (1985)

Limbic, Hypothalamic, Cortical and Spinal Regions are Enriched in Receptors for Thyrotropin-Releasing Hormone: Evidence from [<sup>3</sup>H]ultrafilm Autoradiography and Correlation with Central Effects of the Tripeptide in Rat Brain.

Neurosci. Letts. 60, 337-342

SHIMOHAMA, S., TANIGUCHI, T., FUJIWARA, M. & KAMEYAMA, M. (1985)

Biochemical Characterization of the Nicotine Cholinergic Receptors in Human Brain: Binding of (-)-[<sup>3</sup>H]Nicotine.

J. Neurochem. 45, 604-610

SHIRLEY, S. G. (1987)

A Moving Nozzle Olfactometer.

Chem. Senses 12, 525-536

SHIRLEY, S., POLAK, E. & DODD, G. (1983a)

Selective Inhibition of Rat Olfactory Receptors by Concanavalin A.

Biochem. Soc. Trans. 11, 780-781

SHIRLEY, S., POLAK, E. & DODD, G.H. (1983b)

Chemical-Modification Studies on Rat Olfactory Mucosa Using a Thiol-Specific Reagent and Enzymatic Iodination.

Eur. J. Biochem. 132, 485-494

SHIRLEY, S.G., POLAK, E.H., EDWARDS, D.A., WOOD, M.A. & DODD, G.H. (1987a)

The Effect of Concanavalin A on the Rat Electro-olfactogram at Varying Odorant Concentrations.

Biochem. J. 245, 185-189

SHIRLEY, S.G., POLAK, E.H., MATHER, R.A. & DODD, G.H. (1987b)

The Effect of Concanavalin A on the Rat Electro-olfactogram:  
Differential Inhibition of Odorant Response.

Biochem. J. 245, 175-184

SHIRLEY, S.G., ROBINSON, C.J., DICKINSON, K., AUJLA, R. & DODD, G.H. (1986)

Olfactory Adenylate Cyclase of the Rat. Stimulation by Odorants and Inhibition by Calcium.

Biochem J. 240, 605-607

SHIRLEY, S.G., ROBINSON, C.J. & DODD, G.H. (1987c)

Olfactory Adenylate Cyclase of the Rat.

Biochem. Soc. Trans. 15, 502

SHIRLEY, S.G., ROBINSON, C.J. & DODD, G.H. (1987d)

The Influence of Temperature and Membrane Fluidity Changes on the Olfactory Adenylate Cyclase of the Rat.

Biochem. J. 245, 613-616

SILVER, W.L. & WALKER, D.B. (1987)

Nasal Trigeminal Chemoreception: Response to Nicotine.

Abstract 147 from the 9th annual meeting of the Association for  
Chemoreception Science, Sarasota, Florida

SINGH, P.B., BROWN, R.E. & ROSER, B. (1987)

MHC Antigens in Urine as Olfactory Recognition Cues.

Nature Lond. 327, 161-164

SKLAR, P.B., ANHOLT, R.R.H. & SNYDER, S.H. (1986)

The Odorant-sensitive Adenylate Cyclase of Olfactory Receptor Cells.

Differential Stimulation by Distinct Classes of Odorants

J. Biol. Chem. 261, 15538-15543

SLOAN, J.W., MARTIN, W.R., HOOK, R. & HERNANDEZ, J. (1985)

Structure-Activity Relationships of Some Pyridine, Piperidine, and

Pyrrolidine Analogues for Enhancing and Inhibiting the Binding of

(±)-[<sup>3</sup>H]Nicotine to the Rat Brain P2 Preparation.

J. Med. Chem. 28, 1245-1251

SLOAN, J.W., TODD, G.D. & MARTIN, W.R. (1984)

Nature of Nicotine Binding to Rat Brain P2 Fraction.

Pharmacol. Biochem. Behav. 20, 899-909

SQUIER, C.A. (1986)

Penetration of Nicotine and Nitrosonornicotine Across Porcine Oral  
Mucosa.

J. Appl. Toxicol. 6, 123-128

SYDOR, W., TEITELBAUM, Z., BLACHER, R., SUN, S., BENZ, W. & MARGOLIS, F.L.  
(1986)

Amino Acid Sequence of a Unique Neuronal Protein: Rat  
Olfactory Marker Protein.

Arch. Biochem. Biophys. 249, 351-362

TAYLOR, P. (1980)

Ganglionic Stimulating and Blocking Agents, pp. 211-219 in: The  
Pharmacological Basis of Therapeutics 6th edition, Eds. Gilman, A.G.,  
Goodman, L.S. & Gilman, A., MacMillan

THOMMESEN, G. & DOVING, K.V. (1977)

Spatial Distribution of the EOG in the Rat; A Variation with Odour  
Quality.

Acta. physiol. scand. 99, 270-280

TOTTI, N., McCUSKER, K.T., CAMPBELL, E.J., GRIFFIN, G.L. & SENIOR, R.M. (1984)

Nicotine is Chemotactic for Neutrophils and Enhances Neutrophil  
Responsiveness to Chemotactic Peptides.

Science 223, 169-171

VERNIN, G. (1982)

Heterocyclic Compounds in Flavors and Fragrances Part III. Pyridine and Derivatives.

Perfumer and Flavorist 7, 23-35

VINCEK, W.C., MARTIN, B.R., ACETO, M.D., TRIPATHI, H.L., MAY, E.L. & HARRIS, L.S. (1981)

Synthesis of 4,4-Ditritio-(+)-nicotine: Comparative Binding and Distribution Studies with Natural Enantiomer.

J. Pharmaceutical Sci. 70, 1292-1293

WADDELL, W.J. & MARLOWE, C. (1976)

Localization of Nicotine-<sup>14</sup>C, Cotinine-<sup>14</sup>C, and Nicotine-1' N-Oxide-<sup>14</sup>C in Tissues of the Mouse.

Drug. Met. Disp. 4, 530-539

WADDELL, W.J. & MARLOWE, C. (1978)

Inhibition by Metyrapone of the Accumulation of Nicotine-<sup>14</sup>C in Bronchial Epithelium of Mice.

Arch. Int. Pharmacodyn. Ther 234, 294-307

WAN, K.K. & LINDSTROM, J. (1984)

Nicotinic Acetylcholine Receptor pp.377-430, in: The Receptors, vol.1 ed. Conn, P.M., Academic Press

VEAST, R.C. (1984)

Editor-in-Chief, CRC Handbook of Chemistry and Physics, 65th edition,  
1984-1985, CRC Press Inc. Florida

WILLIAMS, D.C., WHITAKER, J.R. & JENNINGS, W.G. (1985)

Measurement of Nicotine in Building Air as an Indicator of Tobacco  
Smoke Levels.

Environmental Health Perspectives 60, 405-410

WILLITS, C.O., SWAIN, M.L., CONNELLY, J.A. & BRICE, B.A. (1950)

Spectrophotometric Determination of Nicotine.

Anal. Chem. 22, 430-433

WONNACOTT, S. (1986)

$\alpha$ -Bungarotoxin Binds to Low Affinity Nicotine Binding Sites in Rat  
Brain.

J. Neurochem. 47, 1706-1712

WONNACOTT, S., HARRISON, R. & LUNT, G. (1982)

Immunological Cross-Reactivity Between The  $\alpha$ -Bungarotoxin-Binding  
Component from Rat Brain and Nicotinic Acetylcholine Receptor.

J. Neuroimmunology 3, 1-13

WOOD, P. & DODD, G.H. (1984)

Biochemical Studies With Heterocyclic Odorants.

Abstracts of ECRO VI, Lyon, p. 118

WOOD, P., SHIRLEY, S. & DODD, G. (1983)

Effect of Concanavalin A on Frog Olfactory Mucosa.

Biochem. Soc. Trans. 11, 781-782

WYSOCKI, C.J. & BEAUCHAMP, G. (1984)

Ability to Smell Androstenone is Genetically Determined.

Proc. Natl. Acad. Sci. USA 81, 4899-4902

WYSOCKI, C.J., WHITNEY, G. & TUCKER, D. (1977)

Specific Anosmia in the Laboratory Mouse.

Behav. Genetics 7, 171-188

YAMAMURA, H.I. & SNYDER, S.H. (1974)

Muscarinic Cholinergic Binding in Rat Brain.

Proc. Natl. Acad. Sci. USA 71, 1725-1729

## Appendix A.

### Further Information on Nicotine, Calculations and Purity.

#### Industry Standards for exposure to nicotine

Threshold Limit Value = 0.5 mg / cubic metre

for an 8 hour day and a 40 hour week.

Acute Threshold Limit = 1.5 mg / cubic metre

for 15 minutes then no more for one week.

(data from Dr. R. McKeivor, Gallaher Ltd.)

#### Chemical properties of nicotine

The chemical properties of nicotine have been well documented, ranging from Jackson's review of 1941 to an excellent study of 1984 by Seeman which includes data on the orientation and solution conformation of the nicotine molecule. Alkylation of the pyridine ring causes loss or increase in pharmacological potency depending on the ring position of the group and its effect on the rotation of the pyridine moiety around the chiral carbon of the pyrrolidine portion of nicotine (e.g. see Seeman *et al.*, 1985).

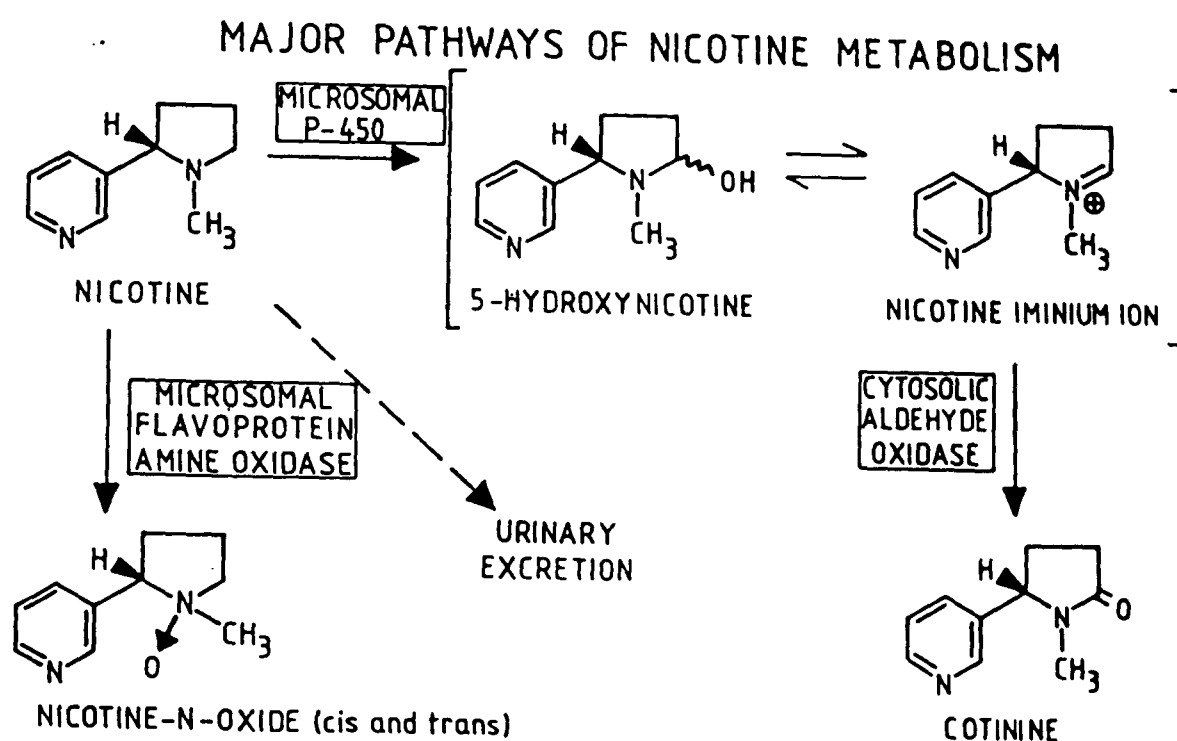
#### Metabolism of nicotine

The major metabolites of nicotine are thought to be cotinine (via a 5'-hydroxy derivative), nicotine-N'-oxide and nornicotine (figure 30).



Cotinine is the major excretory product from nicotine and is used as a marker for exposure to nicotine in clinical tests. Cotinine is thought to be pharmacologically inactive (e.g. see Bowman *et al.*, 1964; Pilotti & Enzell, 1976; Benowitz *et al.*, 1983). Nicotine is metabolised to cotinine in the liver, lung and kidneys but not in the brain (Mangan & Golding, 1984, p.110).

Figure 30.



redrawn from Benowitz *et al.*, 1983 and McCoy *et al.*, 1986

The 5'-hydroxylation of nicotine is mediated by cytochrome P-450 but the conversion of this derivative to cotinine occurs via a cytosolic oxidase. In addition, the conversion of nicotine to nicotine-N'-oxide occurs via a microsomal flavoprotein (Benowitz *et al.*, 1983; McCoy *et al.*, 1986). This N'-oxidase derivative is found in aqueous extracts but can be derivatised to an oxazine which can be extracted from aqueous samples and

analysed chromatographically (Jacob *et al.*, 1986). There is some doubt whether nicotine is demethylated to nornicotine *in vivo* or if traces of this compound are from contaminants in the nicotine used by other workers (McCoy *et al.*, 1986).

#### Calculation of vapour concentration for nicotine

The saturated vapour phase concentration at 15°C (the operating temperature of the odorant delivery system) for nicotine was calculated by interpolation from a plot of log (saturated vapour pressure) versus the inverse of the temperature in degrees Kelvin. These values were obtained from Weast, 1984 (section D-210) and are shown in table 12.

Table 12. Vapour pressure values for nicotine at various temperatures

Temperature (°C)	1/T (10 <sup>-3</sup> /K)	Vapour Pressure (mm Hg)
61.8	2.99	1
107.2	2.63	10
142.1	2.41	40
169.5	2.26	100
219.8	2.03	400
247.3	1.92	760

This gives a value of the saturated vapour pressure for nicotine at 15°C of 0.05 mm Hg. This can be converted to a vapour concentration since

at standard temperature (273°K) and pressure (760 mm Hg) the concentration will be 1/22.4 (M), therefore;

$$\text{vapour concentration at } 15^{\circ}\text{C} = \frac{0.05 \text{ mm Hg} \times 273^{\circ}\text{K}}{760 \text{ mm Hg} \times 288^{\circ}\text{K} \times 22.4} = 2.8 \mu\text{M}$$

This value of 2.8  $\mu\text{M}$  is the saturated vapour concentration above 100% nicotine. To determine the vapour concentrations of nicotine which reach the olfactory mucosa in the electrophysiological experiments, the value was adjusted to account for the concentration of the nicotine in paraffin and the dilution factor in clean humidified air (see Chapter 1, Materials & Methods).

#### Calculation of the water/air partition coefficient.

At 15°C nicotine is fully soluble in water, therefore a plot of vapour pressure versus concentration of nicotine (from 0 to 100%) in water should be linear according to Raoult's Law. The vapour pressure at 100% nicotine converts to the vapour concentration above the liquid as calculated above, thus the partition coefficient can be calculated if the concentration of 100% nicotine is known. This is calculated from the density for nicotine ( $d = 1.01 \text{ g/ml}$ ) to be 6.23 M. The water/air partition coefficient, PC, is;

$$\text{PC} = \frac{\text{Molar concentration in water} \quad 6.23 \text{ M}}{\text{Molar concentration in air} \quad 2.8 \mu\text{M}} = \frac{6.23}{2.8 \times 10^{-6}} = 2200000$$

This means that nicotine vapour will be concentrated by over 2 million fold in the mucus layer (assumed to behave as liquid) at equilibrium.

### Distillation of the nicotine

Distillation of the nicotine was performed under reduced pressure and using extreme caution. Nicotine is very toxic and is absorbed rapidly through the skin (and all mucus membranes). The distillation was done over a one day period in each case with help from Mr. M.A. Wood. Three separate distillations were done during the project with the nicotine being collected at one of the following temperatures (1) 112°C at 9 mm Hg, (2) 116°C at 13 mm Hg and (3) 120°C at 14 mm Hg.

### Purity of the nicotine

The distillation procedure will remove most impurities, for example the residue following distillation will contain most of the cotinine (see Frankenburg & Vaitekunas, 1956) and nornicotine. Some properties of these compounds are shown below.

Table 13. Physical properties of nicotine, cotinine and nornicotine

compound	formula	molecular weight	boiling point (°C)
nicotine	$C_{10}H_{14}N_2$	162.2	247
cotinine	$C_{10}H_{12}N_2O$	176.2	210
nornicotine	$C_9H_{12}N_2$	148.2	270

The redistilled fraction was analysed by capillary gas-liquid chromatography using a Carlo-Erba Fractovap 2450 machine, with an OV-1

column (methyl silicone, internal diameter 0.3 mm, column length 13 metres) and Helium as the carrier gas at 1-2 ml/min. The injector temperature and oven temperature were adjusted to give good resolution of the nicotine peak from the solvent (diethyl ether), leaving sufficient separation to measure any significant impurity peaks. Some examples of the traces obtained are shown in figures 31 and 32. By this analysis the nicotine was determined to be 99.9% pure with the largest impurity at less than 0.1%. No impurity more volatile than nicotine was detected (figure 31). Samples of nicotine were stored in clean glass vials, under nitrogen, protected from the light and at -20°C. Discolouration of the clear liquid was seen with time (several months to a year) to a clear yellow mixture which was found to be over 99% nicotine by capillary GC analysis (see figure 32).

#### UV Analysis

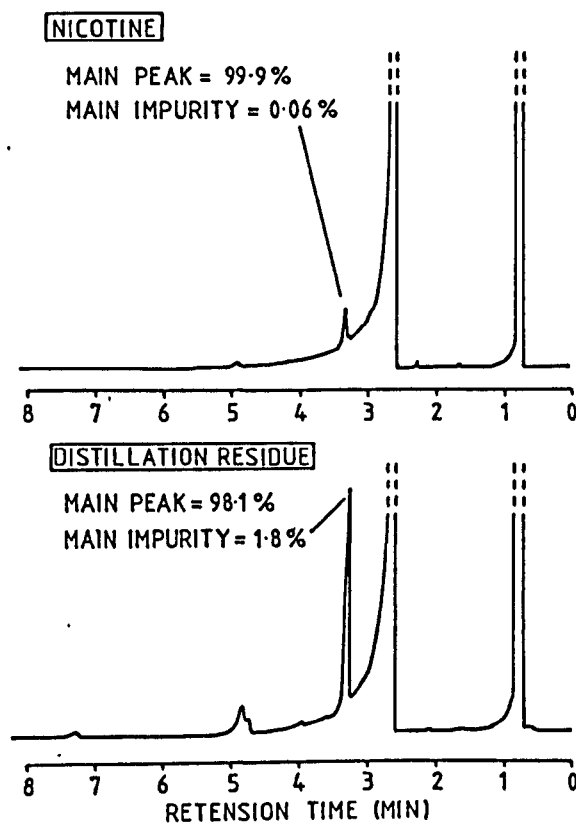
The redistilled nicotine was also analysed by UV spectrometry and gave an absorption pattern (figure 33) expected for the nicotine chromophore (Willits *et al.*, 1950).

#### Optical rotation

The nicotine was found to have an optical rotation of  $[\alpha] = -180$  for a 0.15 M solution in dichloromethane at 23°C. Literature values of  $[\alpha]$  vary from -161 to -170 at 25°C (Weast, 1984; Seeman *et al.*, 1983).

Figure 31. Capillary G.C. Analysis of Nicotine at 142°C

OV-1 COLUMN OVEN-142°C INJECTOR-200°C  
SOLVENT - DIETHYLETHER

Figure 32. Capillary G.C. Analysis of Nicotine at 200°C

OV-1 COLUMN OVEN, INJECTOR - 200°C  
SOLVENT - DIETHYLETHER

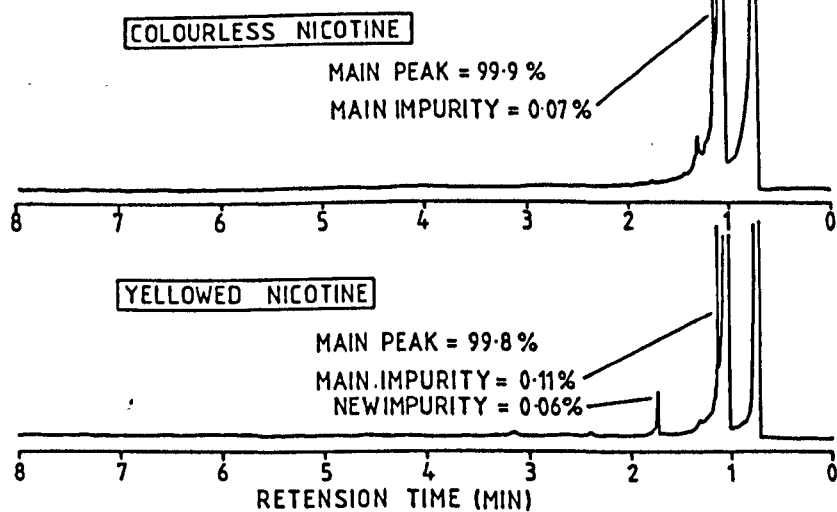
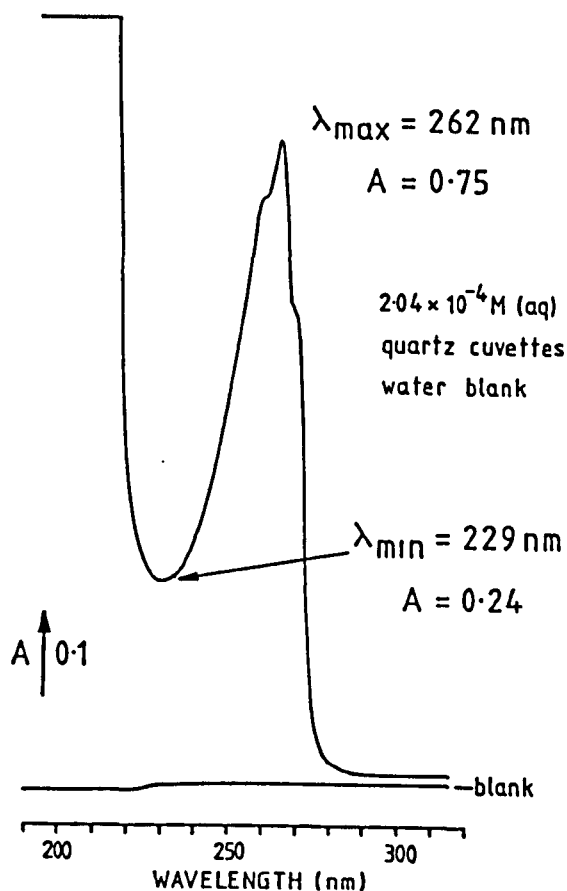


Figure 33. UV Absorbance for Nicotine**Human threshold for nicotine**

An estimate of the detection threshold for nicotine by human volunteers (5 subjects, non-smokers) was made using the redistilled nicotine diluted in ethanol and soaked onto smelling strips. Subjects were asked to identify the smelling strip with nicotine versus a control strip, for a series of binary dilutions from 20% down to 0.15%. In general, the subjects could detect the nicotine at around 2.5% on the smelling strip which corresponds approximately to 70 nM in the vapour phase (this does not allow for evaporation or dilution in air during inhalation).