BIOCHEMICAL SENSING
MECHANISMS IN
OLFACTION

by

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A thesis submitted to the University of Warwick in fulfilment of the requirements for the degree of Doctor of Philosophy

Department of Chemistry

December 1985
For my father, who had such high hopes
and my mother, who is always a great
source of encouragement.
'We are all more influenced by smell than we know'  
(Hercule Poirot) 

from 'Murder in Retrospect' 
Agatha Christie
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ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. G. H. Dodd for his guidance and encouragement throughout the course of this work. In addition, I also wish to express my gratitude to everyone in C221 for their assistance and friendship, particularly Dr. K. C. Persaud and Dr. S. G. Shirley. The technical help provided by Mrs. I. Nealon and Mrs. P. Massey is also appreciated.

Special thanks are due to Dr. P. Pelosi for the donation of radioactive odorant; to Dr. E. Curzon for the recording of high resolution NMR spectra; to Mr. I. Kaytal for the recording of mass spectra, and to Miss U. Knight for her patience and expertise in typing this thesis.

I am also indebted to Prof. K. R. Jennings for permission to use the laboratory facilities of the Department of Chemistry, University of Warwick.

Financial support from the Medical Research Council and the Royal Society is gratefully acknowledged.
DECLARATION

The work described in this thesis was performed unaided by the author unless otherwise stated. No part of this thesis has been previously submitted for a degree from this or any other university.
## ABBREVIATIONS

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<tr>
<td>Å</td>
<td>Angstrom (= 10^{-10} metre)</td>
</tr>
<tr>
<td>b.p.</td>
<td>boiling point</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>Ca^{2+}</td>
<td>calcium ion</td>
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<tr>
<td>cAMP/cyclic AMP</td>
<td>adenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>cGMP/cyclic GMP</td>
<td>guanosine 3',5'-cyclic monophosphate</td>
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<tr>
<td>C.I.</td>
<td>confidence interval</td>
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<tr>
<td>Con A</td>
<td>concanavalin A</td>
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<tr>
<td>c.p.m.</td>
<td>counts per minute</td>
</tr>
<tr>
<td>d.p.m.</td>
<td>disintegrations per minute</td>
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<tr>
<td>D_{2}O</td>
<td>deuterium oxide</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EOG</td>
<td>electro-olfactogram</td>
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<tr>
<td>GDP</td>
<td>guanosine 5'-diphosphate</td>
</tr>
<tr>
<td>GLC</td>
<td>gas-liquid chromatography</td>
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<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineneethanesulphonic acid</td>
</tr>
<tr>
<td>1HMR</td>
<td>proton magnetic resonance</td>
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<tr>
<td>HPLC</td>
<td>high-pressure liquid chromatography</td>
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<tr>
<td>[\textsuperscript{3}H]pyrazine</td>
<td>[\textsuperscript{3}H]2-isobutyl-3-methoxypyrazine</td>
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<tr>
<td>hr</td>
<td>hour</td>
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<tr>
<td>i.d.</td>
<td>internal diameter</td>
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<td>J</td>
<td>joule</td>
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<tr>
<td>mg</td>
<td>milligram</td>
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<td>m.p.</td>
<td>melting point</td>
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<tr>
<td>msec</td>
<td>millisecond</td>
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<tr>
<td>mV</td>
<td>millivolt</td>
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<tr>
<td>m.wt.</td>
<td>molecular weight</td>
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<tr>
<td>Na^{+}</td>
<td>sodium ion</td>
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<td>nm</td>
<td>nanometre</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>pmol</td>
<td>picomole</td>
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<tr>
<td>p.p.b.</td>
<td>parts per billion</td>
</tr>
<tr>
<td>p.p.m.</td>
<td>parts per million</td>
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<tr>
<td>p.s.i.</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>------------------------------------------------</td>
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<tr>
<td>R&lt;sub&gt;F&lt;/sub&gt;</td>
<td>retardation factor</td>
</tr>
<tr>
<td>ROS</td>
<td>rod outer segment</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>T</td>
<td>tesla</td>
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<tr>
<td>T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>spin-lattice relaxation time</td>
</tr>
<tr>
<td>T&lt;sub&gt;2&lt;/sub&gt;</td>
<td>spin-spin relaxation time</td>
</tr>
<tr>
<td>(\tau_C)</td>
<td>correlation time</td>
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<tr>
<td>t.l.c.</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>(\mu)</td>
<td>micron (= 10&lt;sup&gt;-6&lt;/sup&gt; metre)</td>
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<tr>
<td>(\mu l)</td>
<td>microlitre</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
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<td>w/v</td>
<td>weight/volume</td>
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The present work, employing biochemical, biophysical and electrophysiological techniques, attempted to identify specific receptor sites in the vertebrate olfactory system for heterocyclic odorants.

An in vitro rat preparation was developed and characterised for use in vapour-phase chemical modification experiments; the EOG responses obtained from this preparation were stable for up to 5 hours after the death of the animal. The signals to various compounds were differentially reduced when brominated odorants were employed as vapour-phase labelling reagents; the responses obtained to these derivatives and to their non-reactive analogues were preferentially diminished.

The effect of concanavalin A on EOGs obtained from an in vivo frog preparation was examined. This lectin was found to preferentially inhibit the signals elicited by small, sweaty-smelling carboxylic acids; the responses to most of the non-carboxylic acid odorants tested were not significantly inhibited.

The failure to identify specific receptor sites by electrophysiological techniques prompted the performance of odorant binding studies. Examinations of the interaction of [3H]2-isobutyl-3-methoxypyrazine with 15,000 x g supernatant fractions of sheep olfactory epithelium showed that a component of the homogenate fraction exhibited high affinity saturable binding of this odorant (Kd = 10^-6 M). However, the presence of large amounts of non-specific binding substantially decreased the sensitivity and accuracy of the assay. Non-specific binding was observed with tissue fractions of sheep respiratory epithelium, brain and liver. An investigation of binding specificity showed that other bell pepper odorants competed for the 2-isobutyl-3-methoxypyrazine binding site.

The steric requirements for the protein binding of various substituted heterocyclic odorants were examined using nuclear magnetic relaxation techniques. Model studies performed with bovine serum albumin showed that particular side chains of the odorants tested were primarily involved in the binding interaction. The methoxy group of 2-isopropyl-3-methoxypyrazine was found to be responsible for primary recognition by 13,000 x g supernatant fractions of sheep olfactory epithelium.
CHAPTER 1: INTRODUCTION - THE VERTEBRATE Olfactory System

1.1 Morphology

1.1(i) Macroscopic

The olfactory area of the nasal cavity\(^1\) may be readily distinguished from the pinkish colour of the surrounding non-sensory respiratory mucosa by a faint yellow/brown pigmentation\(^2\) (Graziadei, 1971; Dodd and Squirrell, 1980). The importance of this pigment for olfaction has been previously discussed (e.g. see Moncrieff, 1967), although proof of a specific role in the olfactory mechanism (see Section 1.3(ii)b) is still lacking (see Moulton and Beidler, 1967; Moulton, 1971). Its chemical composition is also unclear (but see Kurihara, 1967; also see Moulton, 1971).

The olfactory mucosa of adult vertebrates is composed of a columnar, pseudo-stratified epithelium; a basal lamina, and the lamina propria mucosae. The thickness of the olfactory epithelium varies between animals, ranging from 30\(\mu\) (moles) to 150 - 200\(\mu\) (frogs and turtles). The epithelial surface is usually covered by a layer of mucus (10 - 40\(\mu\) thick) (Graziadei, 1971).

A sagitally-sectioned sheep's head (Figure 1.1) shows the olfactory epithelium to lie upon a series of convoluted structures (turbinates) and on the cribiform plate\(^3\); it is also found on the

\(^1\) No true nasal cavity is observed in fish, where the olfactory epithelium lies in a pair of pits situated in the head (see Graziadei, 1971).

\(^2\) The olfactory region is pale yellow in young cows and turns brownish-yellow with age (Menco, 1977a).

\(^3\) The cribiform plate separates the olfactory area from the brain cavity (Menco, 1977a).
Figure 1.1 A sagitally sectioned sheep's head with nasal septum removed

Taken from Dodd and Squirrel (1960)
posterior portion of the septum (Dodd and Squirrell, 1980; the septum has been removed in Figure 1.1 to reveal the nasal cavities). The turbinates are rigid in order to hold apart the opposing faces of the epithelium. Thus, the system permits a large area of exposed sensory tissue to be packed into a small space (Dodd and Squirrell, 1980). A generally similar basic structure is found for the olfactory region of the rat (Figure 1.2). The number of turbinates and the area occupied by the olfactory epithelium\(^1\) vary throughout the Mammalia (see Table 1.1).

The frog (Figure 1.3) possesses a much simpler nasal cavity than that found in mammals, with no sharp visual delineation being evident between the olfactory and respiratory epithelium. Sensory tissue lines both the ventral part (see Figure 1.3) and the dorsal part of the nasal cavity.

The anatomy of nasal structures from a comparative viewpoint has been described elsewhere (Allison, 1953; Parsons, 1971), whilst Heist et al (1967) have defined in great detail the morphology and localisation of the olfactory epithelium in rabbits\(^2\).

1.1(ii) **Microscopic**

The cellular organisation of the olfactory epithelium (which may be regarded as consisting of three main compartments or regions when viewed in transverse section; Figure 1.4) follows a relatively constant structural pattern in all the vertebrates so far studied (Dodd and Squirrell, 1980). In mammals, the olfactory epithelium

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\(^1\) It should be noted that dimensions of the sensory epithelium are inaccurate as a guide to olfactory acuity since the receptor density varies not only between animal species but also in the same animal as well as between different points of the olfactory epithelium (Graziadei, 1971).

\(^2\) For a brief historical resumé of the histological and morphological elucidation of the vertebrate olfactory mucosa, see Graziadei (1971).
Figure 1.2  Section through the head of a rat

Taken from Burton (1976)
(Also see Figure 2.1)

Figure 1.3  Frog's head after dissection to expose nasal cavity

Taken from Squirrell (1978)
Table 1.1  Comparison of total areas of olfactory epithelium in several species

<table>
<thead>
<tr>
<th>Species</th>
<th>Total Area Of Olfactory Epithelium cm²</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat</td>
<td>13.9*</td>
<td>Negus (1958)</td>
</tr>
<tr>
<td>Felis domesticus</td>
<td></td>
<td>Ferro (1973)</td>
</tr>
<tr>
<td>Dog**</td>
<td>150</td>
<td>&quot; Mulle- (1955)</td>
</tr>
<tr>
<td>Canis familiaris</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frog</td>
<td>ca. 0.5</td>
<td>own observations</td>
</tr>
<tr>
<td>Rana temporaria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Man</td>
<td>2 - 4</td>
<td>Grazisdei (1971)</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>7.27</td>
<td>Heist et al (1967)</td>
</tr>
<tr>
<td>Lepus cuniculus</td>
<td></td>
<td>Mulvkey &amp; Heist (1970)</td>
</tr>
<tr>
<td>Squirrel monkey</td>
<td>3.0</td>
<td>De Lorenzo (1970)</td>
</tr>
<tr>
<td>Saimiri sciureus</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean of 20.3 and 7.0 cm² from Negus and Ferron

** German shepherd dog (Schäferhund).

Taken from Squirrel (1978)
Figure 1.4  Scale drawing of a generalised vertebrate olfactory epithelium

On - olfactory neurone
Sc - supporting cell
Scale: 1 mm = 1 μm

Taken from Dodd and Squirrel (1980)
contains four major cell types (Graziadei and Graziadei, 1978): receptor cells, supporting (or sustentacular) cells, basal cells and the secretory cell of the Bowman's glands. The most peripheral region is mainly occupied by the nuclei of the supporting cells, and generally contains a single nuclear layer (Moulton, 1974). Below this band resides the main compartment of the epithelium, which is predominantly occupied by receptor cell nuclei. The deepest compartment (which extends to the basal membrane) mainly comprises the nuclei of the basal cells. Whilst cells of any type may occasionally be found in any compartment, such exceptions are uncommon and do not invalidate the above categorisations. (Moulton, 1974).

This section briefly describes various ultrastructural or microscopic features of the olfactory neuroepithelium (for review, see Graziadei, 1971; also see de Lorenzo, 1970; Menco, 1977a,b, 1980a,b,c,d; Reese and Brightman, 1970).

1.1(ii)a Olfactory receptor cells

The olfactory neurone is a characteristically shaped bipolar nerve cell, which is located in the epithelium with the terminal swelling protruding at the mucus/cell interface and the axon going to the olfactory bulb via the basal lamina. The cell body is flask-like (ovoid) in shape, with its length being directly proportional to the thickness of the epithelium; the diameter of its processes remains relatively constant (Graziadei, 1971). The terminal swelling

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1 Ciliated respiratory epithelial cells, whilst not part of the olfactory epithelium, are also often closely associated with the tissue (Hirsch and Margolis, 1961).

2 The bulbous ends of bipolar cell dendrites from which arise olfactory cilia "have been misnamed 'olfactory vesicles'" (Reese and Brightman, 1970).
(which is ubiquitously provided with cilia) has a diameter of about 2 - 3µ, the cell body (residing in a rather homogeneous band that occupies the medium third in the height of the epithelium) a diameter of 5 - 8µ and the dendrite 1 - 2µ. Soon after its origin, the unmyelinated axon attains a diameter of 0.2 - 0.3µ which remains constant until it reaches the olfactory bulb (Graziadei, 1971). The receptor cell body contains a large nucleus (4 - 6µ in diameter) which occupies most of its area; the cytoplasm accumulates at the distal pole of the nucleus, whilst only a thin layer (1 - 2µ thick) remains between the nucleus and the membrane.

The density of the olfactory neurones in the epithelium varies according to the species and also according to the location within a particular epithelium; in the cow, it has been correlated with the thickness of the epithelium and shown to be greatest on the septum (Menco, 1977a). The neuronal densities of several animals have been reported (Table 1.2).

1.1(ii)b Cilia

It has long been established that olfactory neurones possess cilia and much speculation has arisen as to their role in the perception of odours (e.g. see Ottoson, 1963; Rhein and Cagan, 1980; but also see Bannister, 1965; Tucker, 1967; see Section 1.3(i)). The cilia arise from the terminal swelling on the apical end of the dendrite; this swelling protrudes slightly above the level of the surrounding supporting cells (Dodd and Squirrel, 1980).

The cilia on olfactory receptor cells possess many common features with other cilia, but some features are unique (i.e. they are sometimes longer than other cilia; Reese, 1965). The proximal portion of the cilia, which possesses the usual "9 + 2" axonemal structure, has a diameter of about 0.25µ; the cilium distinctly
Table 1.2: Comparison of the number of olfactory neurones in several species

<table>
<thead>
<tr>
<th>Species</th>
<th>Total Area of Olfactory Epithelium (cm$^2$)</th>
<th>Density of Olfactory Neurons per cm$^2 \times 10^{-6}$</th>
<th>References</th>
<th>Calculated No. of Olfactory Neurons (millions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat</td>
<td>13.9</td>
<td>9.6</td>
<td>Ferron (1973)</td>
<td>133</td>
</tr>
<tr>
<td>Dog</td>
<td>150</td>
<td>1.5</td>
<td>Muller (1955)</td>
<td>225</td>
</tr>
<tr>
<td>Frog</td>
<td>0.5</td>
<td>1.5</td>
<td>Menco (1977a)</td>
<td>0.75</td>
</tr>
<tr>
<td>Man</td>
<td>2-4</td>
<td>3.0</td>
<td>Kanda et al (1973)</td>
<td>9.0</td>
</tr>
<tr>
<td>Rabbit</td>
<td>7.27</td>
<td>9.0</td>
<td>Heist et al (1967)</td>
<td>65.5</td>
</tr>
<tr>
<td>Squirrel monkey</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* From Table 1.1

Taken from Squirrell (1978)
narrow after a few microns to a diameter of about 0.15 μ and from thence it gradually tapers to about 0.06 μ in diameter (Dodd and Squirrell, 1980). This gradual tapering in the distal region of the cilium is often accompanied by a reduction in the number of microfilaments (Reese, 1965; Frisch, 1967; Thornhill, 1967; Kerjaschki, 1976; Loo, 1977; Kratzing, 1978); as few as two singlet fibres are detected in the distal portions of the olfactory cilia of some species (Kerjaschki, 1976, Loo, 1977; Kratzing, 1978; for review see Steinbrecht, 1969).

Variations between species in the number of cilia per neurone and in their length have been detected. Estimates of the number per receptor have ranged from 1 - 6 for the mole (Graziadei, 1966) to 100 - 150 for the dog (Okano et al, 1967); usually their number varies from 10 - 15 per receptor (Graziadei, 1971). The accurate determination of ciliary length is difficult, since their fragility renders them liable to breakage during microscopic fixation. Consequently, lengths are likely to be underestimated (Dodd and Squirrell, 1980). Reese (1965) reported that the longest frog olfactory cilia extended to a length of up to 200 μ.

Although the respiratory cilia beat in a synchronous wave-like fashion, the active movement of olfactory cilia has been open to dispute (Rhein and Cagan, 1981); any motion that they display (see Hopkins, 1926) has been described as being "uncoordinated and irregular" (Dodd and Squirrell, 1980). Recently, however, Blank et al (1978) reported that olfactory cilia in the frog are motile and that their motility is synchronised by stimulating the mucosa with an odorant. Three types of frog olfactory cilia have been distinguished on the basis of morphology and patterns of movement: "streamers" were comparatively long (30 - 180 μ) and immotile;
"strokers", which moved in a slow whip-like manner, were of intermediate length (15 - 50µ) whilst the shorter "wigglers" (10 - 20µ) made rapid, complex movements (Lair et al., 1980; also see Hopkins, 1926). These cilia types, of which intermediate forms were also observed, occurred in patches across the epithelium.

Some unusual specialisations of the membranes of olfactory cilia have been observed (e.g. see Reese, 1965; Frisch, 1967; Rhein and Cagan, 1981). The presence of large numbers of membrane particles on the freeze-fractured surfaces of sensory cilia from cow (Menco et al., 1976) and mouse (Kerjaschki and Horandner, 1976) olfactory epithelia has been reported. It was postulated that these particles, few of which were detected on the corresponding respiratory cilia, might represent olfactory receptor sites (Menco et al., 1976; see Section 1.3(1)). Subsequent studies have revealed similar particles in the olfactory cilia of the rat, dog, frog (Menco, 1978) and newt (Usukura and Yamada, 1978).

1.1(ii)c Mucus

A layer of mucus overlies the olfactory receptors in all classes of vertebrates; it is thicker and more concentrated in the frog than in mammals (Heist et al., 1967). In amphibians and reptiles, the mucus is secreted partly by the sub-epithelial Bowman's glands and partly by the supporting cells, whilst in mammals and birds, the Bowman's gland is its only source. (Bannister, 1974).

The mucus is motile at the surface: charcoal dust sprinkled on to the surface of the frog olfactory epithelium can be seen to be removed by flowing mucus (Reese, 1965; personal observation). The detailed structure and biochemistry of the olfactory mucus remains generally unknown (Bannister, 1974); in man, the nasal mucus is over 95% water with salts, mucin, neuraminidase and secretory immunoglobulin
present (Abramson and Harker, 1973). It possesses a pH of about 7.0.

The air/mucus partition coefficients for odorants, together with their diffusion rates through the mucus, will control the access of odorant molecules to the receptors (Bostock, 1974); appropriate calculations and experimental measurements suggest that the ligand will rapidly reach the olfactory epithelium (Dodd and Persaud, 1981; also see DeSimone, 1981). The role of the mucus is likely to be the preservation of the necessary ionic environment of the cells; it is improbable that the mucus participates directly in the transduction steps, although it may play a part in olfactory coding (Dodd and Persaud, 1981; also see Section 1.3(iii)). The mucus may also participate in the removal from the mucosa of sorbed odorant molecules (see Hornung and Mozell, 1981).

1.1(ii)d **Supporting cells**

Supporting (or sustentacular) cells are columnar epithelial cells; they possess a larger cell volume than the receptor cells (which they surround at the level of the dendrites) and extend vertically from the epithelial surface to the basal lamina which they reach with branched "digitiform" processes (Graziadei, 1971; Dodd and Squirrell, 1980). The apical surface of the supporting cells is rounded and exhibits a series of irregular microvilli which vary in length (2 - 5 μ long in cat, but can be lacking in other animals) and in density between different animals. Supporting cells provided with cilia have been described in lamprey (e.g. see Thornhill, 1967). The distal parts of supporting cells are approximately cylindrical, whereas they are compressed at the level of the neuronal cell bodies; theirs is the uppermost band of nuclei in the epithelium (Dodd and Squirrell, 1980).
Observations of supporting cells readily reveal the endoplasmic reticulum (both the rough and smooth types) as well as granules of varying size and electron density⁠¹; the release of these granules from the cells may be induced by strong odour stimuli as well as by perfusion of the nasal cavity with dilute solutions of detergent substances (see Reese, 1965; Frisch, 1967; Graziadei, 1971). The specific role of the secretory product and its chemical composition is as yet unknown.

The ratio of supporting cells to neurones is approximately 1 : 1 (Alcock, 1901; Heist et al, 1967).

1.1(ii)e Basal cells

The layer of nuclei residing deepest in the olfactory epithelium belongs to the basal cells. These prismatic cells are located close to the epithelial surface of the basal lamina and arranged between the basal processes of the supporting cells; they ensheath groups of axons (Graziadei, 1971; Dodd and Squirrell, 1980). Their function, other than that of replacement elements, is as yet unclear.

1.1(ii)f Olfactory axons

The axons extend from the cell bodies of the olfactory neurones to the glomeruli of the olfactory bulb without synapsing (Dodd and Squirrell, 1980). They run for a short distance inside the epithelium and emerge grouped into small bundles of a few tens of units; after crossing the basal lamina, these bundles (15 - 40 at a time) are ensheathed by Schwann cells (Gasser, 1956; also see de Lorenzo, 1963). They then turn to run parallel to the basal membrane, all the while

⁠¹ E.g. electron micrographs of frog supporting cells consistently show the presence of large secretory droplets (see Yamamoto et al, 1965); similar observations have been made on the newt (see Usukura and Yamada, 1978).
being grouped into progressively larger bundles, before changing
direction to assume a vertical course through the cribiform plate to
the brain (Dodd and Squirrell, 1980). The mean diameter of the
axons is 0.2 μ (Graziadei, 1971).

In the rabbit, it has been estimated that each glomerulus
is, on average, connected to 25,000 axons (Allison and Warwick, 1949).

1.1(ii)g The lamina propria

The lamina propria separates the olfactory epithelium from the
underlying bone. It consists of connective tissue, olfactory nerve
fibres, a vascular system and the mucus-secreting Bowman's glands
whose ducts extend to the epithelial surface (Dodd and Squirrell, 1980).

1.1(ii)h Bowman's glands

Bowman's glands, which are found only in the olfactory region
of all vertebrates except fish, are tubular alveolar units. Their
histological composition is quite similar among species, although the
ultrastructural details of the secretory cells vary not only between
species but also within species, where cells possessing a variable
organelle content have been observed (see Graziadei, 1971).

1.1(ii)i Contacts between cells

If the olfactory epithelium is sectioned parallel and close
to the epithelial surface, the terminal swellings of the dendrites
may be observed to be spaced apart by the supporting cells which
possess a larger cross-sectional area; at this level cells are
connected to each other by a tight-junctional belt\(^1\) (Dodd and

\(^1\) Two types of tight junction have been recognised (Farquhar and
Palade, 1963; Revel and Karnovsky, 1967): true tight junctions
occlude extracellular spaces, whilst gap junctions appear to be
sites of cell-to-cell electrical coupling (e.g. see Brightman and
Reese, 1969). It has been shown that true tight junctions are
present in the olfactory epithelium of animals such as the frog
(Brightman and Reese, 1969), the monkey and the elasmobranch
(Reese and Brightman, 1970).
Squirrell, 1980). It has been demonstrated in the elasmobranch that intravenously injected horseradish peroxidase (molecular weight: 42,000) cannot diffuse past these junctions to the epithelial surface\(^1\) (Reese and Brightman, 1970). Hence, the tight junctions prevent diffusion between the mucus and the inter-cellular spaces within the epithelium; they may also provide the epithelium with mechanical strength (Dodd and Squirrell, 1980).

Contacts between the neurones occur only occasionally at the level of the dendrites and terminal swellings, although the cell bodies frequently touch one another (Dodd and Squirrell, 1980).

1.1(ii)j The vomeronasal epithelium

The nasal cavities of amphibians, reptiles and mammals contain, in addition to the olfactory epithelium proper, a distinct sensory area known as Jacobson's organ or vomeronasal epithelium\(^2\). This epithelium, which responds to stimuli in a similar fashion to the olfactory organ proper (see Tucker, 1963b, 1971; also see Beauchamp et al, 1984), contains primary receptor neurones whose axons terminate in the accessory olfactory bulb. Although the vomeronasal epithelium exhibits many embryological, anatomical and functional similarities with the olfactory epithelium, some characteristic differences have been noted\(^3\); its ultrastructural details, based on the observations of some reptiles, have been described elsewhere (Graziadei, 1971).

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\(^1\) Some tight junctions have been shown to be impervious to molecules of molecular weight as low as 1800 (Feder et al, 1969) or possibly even smaller (Farquhar and Palade, 1965).

\(^2\) The organ of Jacobson is absent or rudimentary in the human adult, although it is clearly demonstrable during intrauterine development (Tucker, 1971).

\(^3\) E.g. the vomeronasal receptors may contain no cilia, but do possess microvilli (Beidler, 1970).
Structural aspects of ageing in the mouse vomeronasal (and olfactory) epithelium have recently been discussed by Dodson and Bannister (1980).

1.1(iii) Cell dynamics in the olfactory epithelium

The olfactory organ (unlike most receptor sheets) is directly exposed to airborne chemicals; this creates vulnerability to bacterial and viral invasion, to the mechanical impact of the respiratory airstream, and possibly to the toxic effects of environmental pollutants. However, despite this exposure, the olfactory system of most individuals appears to retain its sensory capabilities throughout life, thereby suggesting that the system possesses specific mechanisms for maintaining function in the face of cell loss (see Moulton, 1974).

Dividing cells in adult olfactory epithelia have been observed by many investigators. An early belief held that basal cells divide continuously, with the products replacing supporting cells or the cells of Bowman's glands. However, mitotic activity has also been noted in other cell types of the epithelium, including the receptor cells (see Moulton, 1974). Andres (1970) detected mitoses in the basal and supporting cells, but only sparingly in the sensory cells. In addition, he described a fourth cell-type ("Blastenzelle") lying beneath the basal cells in the lamina propria of rats, cats and dogs which he originally believed to be a reservoir for the differentiation and renewal of the sensory receptors; it was later concluded, however, that although these cells were

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1 Rhinitic infections, with accompanying pathological changes in the olfactory epithelium, are common in at least some species of laboratory animals (Moulton, 1974).

2 Since the number of olfactory receptors is great (10⁶ in man; 10⁸ in rabbits) it is feasible that even extreme attrition need not severely impair function (Moulton, 1974).
common in young animals, they were rare in adults (Andres, 1970).

Quantitative observations of mitotic activity in the olfactory epithelium have been made by administering a radioactive label (e.g. tritiated thymidine) to the experimental animal such that the products of cell division remain identifiable (e.g. with autoradiographic techniques). Utilising such an approach, the basal cells of mouse olfactory epithelium have been shown to be continuously dividing (see Leblond and Walker, 1956); the products of division migrated peripherally, with the majority of the migrating cells remaining in the receptor cell compartment. It was thus concluded that receptor cells undergo continuous renewal (Moulton et al, 1970; Moulton and Fink, 1972).

Studies on frogs led to a similar conclusion; labelled nuclei were found at all levels in the epithelium. Some were identified as being those of neurones, whilst others were of supporting cells, basal cells and cells in intermediate stages of development. Inconclusive evidence was presented in favour of basal cells giving rise to supporting cells (Graziadei and Metcalf, 1971; Graziadei, 1973).

In contrast to these findings was the observation that few labelled cells were detected in the olfactory epithelium of the lamprey less than 24 hours after injecting tritiated thymidine (Thornhill, 1970). However, after five days "fairly large" numbers of labelled nuclei were observed. Evidence for the continuous renewal of receptors in the trout epithelium has also been reported (Bertmar, 1973).

A possible criticism of studies exploiting thymidine is that cells may incorporate thymidine while undergoing repair instead of dividing; generation times may also be influenced by the
administration of label. Although turnover times obtained with this and other methods display general agreement, it is valuable to confirm critical findings by an independent method (Moulton, 1974). One such approach involves the use of colchicine (a plant alkaloid), which blocks cells undergoing division in metaphase. A comparison of the number of blocked metaphases that have accumulated three and five hours after colchicine injection provides an estimate of turnover times; in mice, when averaged over the entire epithelium, estimates of about 30 days$^1$ were obtained (Moulton, 1974, 1975). However, much of the activity was attributable to the more rapid turnover time of the basal cells (8 - 16 days assuming no cell loss from this compartment), thus confirming that in the mouse they form a renewing population$^2$ (Moulton et al., 1970). It should be noted that the colchicine method furnishes no information about cell migration.

1.1(iv) Experimentally-induced degeneration and subsequent regeneration of the olfactory epithelium

Associated with the ability of the olfactory epithelium to undergo a continuous process of cell renewal is its ability to regenerate following destruction of the nerve cell population. In agreement with the generally held belief that neurones in adult vertebrates are incapable of regeneration (Moulton, 1974; Dodd and Squirrel, 1978), many investigators have found no evidence for such reformation (see Moulton, 1974). However, some studies have indicated that regeneration does occur. The earlier investigations have been

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1 30 days is relatively long compared with certain other tissues, e.g. taste buds ($10 - 10^2$ days; Beidler and Smallman (1965)), or the epithelium of the small intestine (1 - 3 days); for discussion, see Moulton (1974).

2 Graziadei (1973) found that, in the frog, the rate of turnover of neurones is greater than that of the supporting cells.
extensively reviewed by Takagi (1971) who considered it "highly conceivable" that new olfactory neurones could be generated from undifferentiated stem cells in order to enable the tissue to recover from insult. Regeneration has now been demonstrated in such species as fish (Westerman and Von Baumgarten, 1964; Cancalon, 1980; Zippel and Breipohl, 1980), frog (Graziadei and Metcalf, 1971; Graziadei and DeHan, 1973; Mair et al, 1980), mouse, rhesus monkey, rabbit (e.g. see Mulvaney and Heist, 1971; Matulionis, 1975; Graziadei, 1978), pig (Baldwin et al, 1980) and pigeon (Tucker et al, 1975; Bedini et al, 1976).

Most studies of regeneration involve experimentally-induced degeneration of the epithelium or receptors. The olfactory receptors appear to degenerate when any one of a number of procedures is applied, including severance of the primary neurones in fish, frog, guinea pig and mouse (e.g. see Takagi and Yajima, 1964, 1965; Graziadei and Metcalf, 1971), administration of colchicine in rabbits (Jackson and Lee, 1965), partial or complete ablation of the olfactory bulb in fish (Zippel and Breipohl, 1980), pig (Baldwin et al, 1980), rabbit and rat (e.g. see Sen Gupta, 1967; Graziadei, 1978) and application of a 15% zinc solution in frog, mouse, rat, rabbit or rhesus monkey (e.g. see Mulvaney and Heist, 1971; Matulionis, 1975).

Although the receptors appear to degenerate rapidly and completely with all these procedures, the various techniques may induce different degrees of epithelial degeneration (Moulton, 1974): nerve sectioning or olfactory bulb ablation, which may leave non-receptor elements apparently intact or hypertrophied, causes retrograde degeneration of all axons and neurones in the epithelium within a few days. The basal cells, which remain along with the supporting cells, are stimulated into intense activity by this degradation; they
divide and differentiate such that the epithelium is reconstituted within a few weeks, the axons having grown back to make connections with the glomeruli of the olfactory bulb.

The necrotic destruction of the epithelium by administration of zinc sulphate can be relatively complete, destroying all cells down to the level of the lamina propria\(^1\) (e.g. see Mulvaney and Heist, 1971). Nevertheless regeneration still occurs (within 30 days in the rabbit, and within 6 months in the rhesus monkey); in rats, the ability to perform in an odour detection task is restored in about 5 - 7 days (see Moulton, 1974).

The degeneration and subsequent regeneration of pigeon olfactory neurones (Tucker et al, 1975) have been shown to be accompanied by behavioural and electrophysiological changes which parallel the changes in cell structure (also see Baldwin et al, 1980). Recently, Mair et al (1980) have reported that when the frog olfactory epithelium is ablated by irrigation with zinc sulphate (0.1M), neurones regenerate and cilia reappear over an eight week period; "wigglers" and "strokers" (see Section 1.1(ii)b) are first identified at 2 weeks after treatment, and at 4 weeks after treatment, the first "streamers" are apparent in localised patches. This pattern of ciliogenesis is consistent with the hypothesis that "wigglers" and "strokers" are early developmental stages of "streamers".

\(^{1}\) It may be difficult to ensure that islets of undamaged tissue do not persist with this method (see Moulton, 1974).
1.2 Electrical activity in the olfactory epithelium

The interaction of odorous stimuli with the receptor cell membrane may be signalled "by electrical activity from the olfactory neurones" (Dodd and Squirrell, 1980; also see Getchell and Getchell, 1974). The techniques used to study such activity may be divided into two large groups: single unit techniques, employing various microelectrodes and recording both intra- and extracellularly, distinguish the activity of single nerve cells or of a few nerve cells that are electrically close to each other; ensemble techniques primarily measure the properties of a population of neurones and consequently provide little information about how any particular cell responds (see Gesteland, 1971).

The most commonly used ensemble technique involves recording from either a whole nerve or a filament of the olfactory nerve, the accessory olfactory nerve or the trigeminal nerve (e.g. see Mozell, 1964b, 1966; Tucker, 1963a, b). An alternative method is to record the potential at the surface of the olfactory bulb where the principal component of the voltage recorded is due to activity in the terminals of the olfactory receptor cells (Ottoson, 1959a, b, c). The third ensemble signal event which may be recorded is called the electro-osmogram or electro-olfactogram (EOG) in vertebrates and electro-antennogram (EAG) in insects. Both of these signals are causally related to the generator events, but "can hardly be thought to be the generator event itself" (Gesteland, 1971). The EOG is recorded by placing a reversible electrode on the surface of the olfactory mucosa and measuring the voltage between it and an indifferent electrode located elsewhere on the animal (see Hosoya and Yoshida, 1937; Ottoson, 1954).

Some of the characteristic electrical signals obtained from the olfactory epithelium are shown in Figure 1.5.
Figure 1.5  Diagrammatic representation of electrical activity recorded from various parts of the olfactory epithelium in response to an odorant

A. Electro-olfactogram from Gesteland et al., (1965)
B. Excitatory single-cell impulse activity based on van Drongelen, (1978)
B'. Impulse activity on an expanded time scale from Gesteland et al., (1963)
C. Impulse activity in the olfactory nerve based on Shibuya, (1964)
D. Summated neural discharge based on Tucker and Shibuya, (1965)

The olfactory neuron on the left is drawn to show the positions from which the responses are obtained.

Taken from Squirrell (1978)
1.2(1) The electro-olfactogram (EOG)

Following the observation that the odorous stimulation of rabbit (Ottoson, 1954) or frog olfactory membranes (Ottoson, 1956) gave rise to a slow sustained potential, the term electro-olfactogram, or EOG, (c.f. the retinogram in the simple invertebrate eye) was introduced (Ottoson, 1956) to designate "the monophasic negative potential evoked by odours in the sensory region of the nasal mucosa" (Ottoson, 1971). It was later noted (Takagi and Shibuya, 1959) that this mucosa potential had first been recorded in 1937 from canine olfactory epithelium (Hosoya and Yoshida, 1937).

EOG responses have now been recorded from many other sources, including fish (e.g. see Shibuya, 1960; Tucker and Shibuya, 1965) and other amphibians such as the newt (Kauer and Moulton, 1974); from reptiles (e.g. turtle (Tucker, 1963b); eel (Silver, 1980)); from birds (Shibuya and Tucker, 1965) and from mammalian species such as the rat (e.g. see Gesteland and Sigwart, 1977; Persaud, 1980; Shirley et al, 1980, 1981, 1983a,b, 1984a), tortoise, guinea-pig (MacLeod, 1959), pig (T. M. Poynder, personal communication), sheep (Squirrell, 1978) and man (Osterhammel et al, 1969; also see Plattig and Kobal, 1978). Thus EOGs appear to be a general feature of olfactory epithelia.

The electrical response of the frog's olfactory epithelium to a short puff of odorous air is a slow negative monophasic potential with a steep rising phase (i.e. short rise time) which is followed by an exponential return towards the baseline (Figure 1.6; Ottoson, 1971). It is now generally accepted that this "negative-on" EOG accurately

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1 For a review of studies on the receptor potential of the olfactory organ, see Ottoson (1971); also see Ottoson (1970).
Figure 1.6  

The electro-olfactogram

Superimposed records of responses of frog's olfactory organ to brief stimulations with butanol-vapour of different strengths.

Taken from Ottoson (1956)
reflects the activity of the olfactory receptor cells (i.e. it represents the summated generator potentials of the olfactory neurones). The evidence for such a view may be summarised as follows:

(a) The EOG peak amplitude is proportional, within limits, to the logarithm of the stimulus concentration (i.e. it is a stimulus-related, graded response) (Figure 1.7; see Ottoson, 1956; Poynder, 1974a,b; Gesteland and Sigwart, 1977).

(b) The shape of the EOG response is similar to that of the generator potentials observed in other sensory receptors (Figure 1.8).

(c) The EOG is not simply a product of the activity of the nerve fibres; anti-dromic stimulation of the olfactory nerve does not bring about a potential change at the mucus level (Ottoson, 1956; 1959b).

(d) EOG generation is mainly dependent upon the entry of sodium ions into the receptor cells (Takagi et al, 1966, 1968, 1969a; see Figure 1.9; also see Section 1.2(iv)); such a mechanism is normal for depolarising generator potentials.

(e) The EOG is not abolished by cocaine at concentrations sufficient to block the impulse activity of the olfactory nerve (Ottoson, 1954, 1956; Kimura, 1961); such resistance is a general feature of generator potentials (e.g. the generator potential recorded from frog muscle spindle (see Figure 1.10; Ottoson and Shepherd, 1965; also see Eyzaguirre and Kuffler, 1955)).

(f) EOG responses are only obtained from regions of the mucosa lined with sensory cells (see Figure 1.11; Ottoson, 1956, 1971; Takagi and Yajima, 1964, 1965).

(g) The EOG is accompanied by a change in transepithelial impedance (see Figure 1.12; Gesteland et al, 1965). This is to be expected
Figure 1.7  Variation in EOG peak amplitude with logarithm of ethyl n-butyrate vapour concentration

Vertical bars indicate range of values measured for 10 animals (rats).

Taken from Gesteland and Sigwart (1977)
Figure 1.8  A comparison of receptor potentials recorded from various sources

a. Mechanoceptor
b. Photoreceptor
c. Chemoreceptor
d. Thermoreceptor

Eledone: ERG
--- 1 sec

Frog: EOG
--- 5 sec

a. frog muscle spindle
b. blowfly retinula cell
c. antenna of male cockroach
d. infrared receptor

ERG from eye of Eledone
EOG from the olfactory organ of frog

Taken from Ottoson (1974)
Also see Fuortes (1971)
Figure 1.9  
ECG responses to menthone vapour in Na⁺-free Ringer solution

1 shows the ECG in normal Ringer solution

2-9 show records taken at successive 20 minute intervals from excised frog epithelium immersed in Na⁺-free solution

Taken from Takagi et al (1968)

Figure 1.10  
The receptor potentials from frog muscle spindle

(a) with impulse activity superimposed
(b) after the conducted activity has been blocked by 0.2% lignococaine.

Time bar: 50 milli-seconds

Taken from Ottoson and Shepherd (1965)
Figure 1.11 Schematic diagram showing responses to stimulation of restricted areas of frog olfactory mucosa

Dotted area indicates sensory epithelium.

Taken from Cttoson (1956)

Figure 1.12 The EOG and the variations in transepithelial impedance during odorant stimulation

Sweep length: 10 seconds

Taken from Gesteland et al (1965)
since a change in membrane permeability is a pre-requisite for the flow of a generator current.

(h) Since a generator potential is the causal event for a chain of activity at higher levels, the EOG must be shown to possess a direct relationship with such higher activity:

(i) The EOG is related to the action potentials recorded from single cells in the olfactory epithelium (see Figure 1.13; Gesteland et al, 1963).

(ii) Impulse activity in the olfactory nerve closely follows the EOG response (see Figure 1.14; Kimura, 1961; Tucker, 1965b; Shibuya, 1964; also see Døving, 1964); simultaneous recordings of the EOG, spike activity in the epithelium and nerve discharge evoked by various concentrations of amyl acetate show that the onset of the EOG precedes these other activities (Figure 1.15; Shibuya, 1969). If the EOG is to represent a generator potential, which is causative of impulse activity, then such behaviour is essential.

(iii) The relationship between the ECG response and the slow potential from the olfactory bulb (Ottocon, 1959b), or the activity from bulbar units (Døving, 1966) is shown in Figures 1.16 and 1.17. In the bulb, both excitation and inhibition of impulse activity is observed.

(iv) The EOG recorded from humans is matched by the psychophysical response (Flattig and Kobal, 1978).

Despite these arguments, experimental evidence against the ECG representing the generator potential has been presented. Shibuya (1964) recorded the impulse activity of a small strand of the tortoise olfactory nerve. A small piece of adsorbent paper was then placed onto the area of the olfactory membrane innervated by the fibres and
Figure 1.13  The ECG and single unit activity from the frog

Taken from Gesteland et al (1963)

Figure 1.14  The EOG and impulse activity in the olfactory nerve

(a) The EOG superimposed upon impulse activity in the olfactory nerve.

Taken from Shibuya (1964)

(b) The upper traces show the EOG and the lower traces the summated neural discharge. The fractional saturation of amyl acetate as stimulus is indicated.

Taken from Tucker (1963b)
Figure 1.15
Simultaneous recording of EOG, single cell spike activity and olfactory nerve discharge

Figure 1.16
Comparison of the EOG with the slow bulbar potential for responses to butanol

The ECG is the lower trace. A - C indicates increasing concentrations of butanol. Vertical bars represent 1 millivolt; the horizontal bar is a 1 second time mark.

Figure 1.17
The effects of olfactory stimulation on bulbar units and the EOG

Unit A shows excitatory activity whilst the firing of Unit B is inhibited.
following its removal, the EOG in the treated area was found to have disappeared although impulse activity could still be recorded from the strand. This led to the conclusion that the EOG did not represent the generator potential (but see Ottoson, 1963; Ottoson and Shepherd, 1967).

"Some of the most obvious deficiencies of Shibuya’s experiments and conclusions" have been previously discussed by Ottoson (1971). It was suggested that the remaining impulses may have derived from fibres coming from regions outside the treated area; the effect of subjecting the entire sensory epithelium to treatment had not been investigated (Ottoson, 1971). When this effect was later examined (Ottoson and Shepherd, 1967), both the EOG and the afferent discharge were found to disappear. Claims that the disappearance of the EOG responses was due to removal of the mucus (Shibuya, 1964) have also been refuted and the study re-interpreted as providing "an elegant demonstration" that the olfactory cilia represent the chemosensitive elements of the receptor cell (Ottoson, 1971; see Section 1.3(1)); the EOG disappeared because of the destruction of the cilia (see Ottoson, 1970). Takagi (1967) has further suggested that the epithelial treatment might have succeeded in removing water from the mucus, thereby changing the mucus resistance and precluding the successful recording of EOG responses.

Studies by Mozell (1962) have also been cited as evidence against the generator hypothesis; they indicated that the EOG response was not always matched by the summated afferent discharge. However, it has been emphasised that "the differences occurred only under very specific conditions that had to be isolated from the many conditions under which the two paralleled each other"; that the summated discharge might be suspect since it depended "not simply on the spike
frequency but also on the spike amplitude", and that "the neural responses might not have been elicited by the same mucosal regions which yielded the ECG" (Mozell, 1962).

Further doubts about the generator hypothesis have been raised by the finding that the bulbar waves did not always conform to the positive potential or the off-effects (see Section 1.2(ii)) produced in the receptor layer with ether or chloroform; in addition, the EOG did not always increase with raised odorant concentration (Takagi, 1967; for review see Takagi, 1969). However, since the potentials evoked with substances such as ether differ greatly from one region of the sensory epithelium to another¹ (Takagi and Shibuya, 1960a,b), Ottoson (1971) argued that the presence or absence of conformity between the peripheral response and the bulbar waves would depend upon the area of the mucosa or the bulb from which the recording was made.

The limitations of the EOG as an investigative tool have recently been considered by Mair and Gesteland (1980): the ECG, which is not simply related to the response properties of any particular cell, was noted to be an insensitive measure and therefore not useful for measuring the responses near threshold nor for comparing

¹ Important evidence has been acquired from EOG studies which supports the hypothesis that different regions of the receptor epithelium are particularly sensitive to different odours (e.g. see Daval and Leveteau, 1969; Daval et al, 1972; Mustaparta, 1971; Kauer and Moulton, 1974; Blank and Mozell, 1981; Mackay-Sim et al, 1982). Two mechanisms ("imposed patterning" which is dependent upon nasal patency, nasal air flow and the relative air/mucus solubility of an odorant (see Section 1.3(iii)); and "inherent patterning" which is dependent upon receptor cells of similar sensitivities being grouped together) have been proposed to account for odour discrimination on the basis of spatial effects. These mechanisms are not necessarily mutually exclusive; indeed, "if present, they may complement each other, and there is now evidence for both" (Kubie et al, 1980).
olfactory sensitivities to different substances. However, EOG studies do "provide information from which physiological mechanisms for receptors can be inferred", although they "have not yet provided much information about how one odour is distinguished from another".

1.2(ii) Positive EOGs and other potentials

It has been suggested that the term electro-olfactogram (EOG), initially proposed for the negative voltage transient (Ottoson, 1956), should be broadened in definition and meaning to include any initial positive voltage component\(^1\) (Gesteland, 1964; but also see Shibuya, 1964; Tucker and Shibuya, 1965; Ottoson and Shepherd, 1967; Takagi, 1969; Ottoson, 1971; Gesteland, 1971).

A variety of slow potentials have been elicited from the olfactory epithelium under appropriate conditions (Figure 1.18) (see Takagi and Shibuya, 1959; Higashino and Takagi, 1964; Takagi et al, 1966, 1968; Takagi, 1969). These waveforms may be explained by postulating that "a particular stimulus can evoke both excitatory and inhibitory processes and that the time constants of each process are independent of each other and stimulus-related" (Gesteland, 1971).

The frequency of occurrence of the EOG waveforms has been examined by Takagi et al (1969b); in a survey of 122 different odorants, the negative-on EOG was found to be by far the most common type produced (obtained to 87% of the odours). Consequently, it was concluded that all but this type of EOG response are exceptional and unphysiological (Takagi et al, 1969b).

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\(^1\) E.g. the slow voltage change evoked by 1-butanol exhibits a small positive transient \((V_{\text{EOG}(+)}\)\) followed by a larger amplitude negative voltage component \((V_{\text{EOG}(-)}\) \(\) (Getchell and Getchell, 1974).
(1) A "negative-on" EOG

(2) A monophasic positive EOG. This "positive on" EOG was first studied by Takagi et al. (1960), although Ottoson (1956) had occasionally observed an initial positive deflection preceding the negative EOG which he believed to be an artifact caused by positively charged water ions.

(3) A "positive off" EOG. This positive-going transient which appears at the cessation of stimulation was discovered by Shibuya (1960); other examples may be found in Gesteland et al. (1965).

(4) A "negative-off" EOG. This type of response was first described by Takagi and Shibuya (1959), working on frogs. The example shown here also displays a small positive-on wave.

(5) A "positive-after potential" obtained from rabbit. This was first reported by MacLeod (1959).
Figure 1.18  Potentials that can be elicited from the olfactory epithelium

(1)  
10 seconds

Negative-on EOG  Animal: frog  
Odorant: pyridine  
From Gesteland et al., (1963)

(2)  
5 seconds  
From: Gesteland et al., (1965)  
Positive-on EOG  Animal: frog  
Odorant: methanol

(3)  
5 seconds  
Animal: Channa argus  
Odorant: pupa fluid  
Positive-off EOG  From: Shibuya, (1960)

(4)  
2 seconds  
Animal: frog  
Odorant: diethyl ether  
Negative-off EOG  From: Takagi et al., (1960)

(5)  
30 seconds  
Positive-after Potential  Animal: rabbit  
Odorant: benzene  
From: MacLeod, (1959)

Vertical scale: millivolts, negative upwards; horizontal scale: time in seconds as indicated. 
Stimulus indicated by thick bar or arrow.
1.2(iii) **Cellular origins of electro-olfactograms in the olfactory epithelium**

The experimentally-induced degeneration of the neuronal cell population in the olfactory epithelium (see Section 1.1(iv)) has formed the basis of several investigations into the cellular origins of slow potentials; following the unilateral sectioning of frog olfactory nerves, the observed electrical responses were compared with the histological changes in the degenerating and normal olfactory epithelium (Takagi and Yajima, 1964, 1965; Takagi and Wyse, 1965; Takagi et al, 1969b; for review see Takagi, 1971).

The negative EOG response was found to disappear along with the degenerating neurones, whereas the positive EOG not only survived but was often more pronounced; the negative parts of complex responses disappeared, but the positive parts persisted (Figure 1.19).

These observations suggested that the negative EOGs originate in the olfactory receptor cells and that the positive potentials arise from the supporting cells (Takagi, 1967). The basal cells may be excluded from consideration because of their deep position in the epithelium; the latency\(^1\) of the EOG response is much too short to allow for the diffusion of odorants to them.

1.2(iv) **Ionic mechanisms**

The ionic basis of EOG generation has been investigated by examining the effects of variously-composed Ringer solutions on the potentials obtained from excised frog olfactory epithelium (Takagi et al, 1966, 1968, 1969a,b). It was found that the negative EOG depended mainly upon the influx of sodium ions, accompanied by an increased membrane permeability to potassium ions, whilst positive potentials (which were compared with the inhibitory potentials of other receptors) were principally determined by the movement of

\(^1\) About 200 milliseconds (Cotton, 1956).
Changes in amplitude of the EOG's. In each pair of records, the slow potential at the top was obtained as a control in the olfactory epithelium of the normal (left) side and the potential at the bottom was recorded in the sectioned (right) side of the same bullfrog. The responses were obtained 5 days after the nerve section in a, 6 days after in b, 7 days after in c, and 8 days after in d. Here the EOG completely disappeared. In e, f, and g, which were obtained 9, 10, and 12 days after respectively, no response could be found in the sectioned side (at the bottom).

Taken from Takagi and Yajima (1965)

Negative and positive EOGs. The negative and positive EOGs elicited in the normal olfactory epithelium are shown on the left and the ones elicited in the degenerated epithelium are shown on the right. It is clear that the negative EOGs are not elicited at all or very small artifact potentials are produced in the degenerated epithelium. The negative off-EOG elicited by chloroform (Cl) vapour also disappears in the degenerated epithelium and only a vestige is seen as indicated by an arrow (bottom records). AA, amyl acetate. The horizontal bars at the bottom indicate 4 sec.

Taken from Okano and Takagi (1974)
chloride ions, again with a contribution from potassium ions. The ionic basis of the positive EOG response was observed to be identical in normal and degenerating epithelium.

Electron microscopy has been used to examine the effects of odour stimulation at a cellular level; when chloroform vapour, a typical positive EOG-generating odorant, was applied to degenerating and degenerated olfactory epithelia, granules were secreted from the supporting cells (see Section 1.1(ii)a) much more vigorously than when amy1 acetate or other negative EOG-eliciting compounds were similarly applied (Takagi, 1969, 1971; Okano and Takagi, 1974). Consequently, it was thought "very probable" (Takagi, 1971) that at least most of the positive EOGs (i.e. the positive after-potential discernible only after the negative EOG has been switched off) are generated by this secretory activity of the supporting cells.

In summary then, complex EOG waveforms may be thought to reflect a combination of the negative generator potential, a hyperpolarising positive potential and a slow positive secretory potential.

1.2(v) **Generator currents in the olfactory epithelium**

The mechanism by which nerve impulses are generated in the olfactory cells is thought to be similar to other neurones (Dodd and Squirrell, 1980). In short, odorous stimulation effects the opening of ion-gates in the sensory region of the neuronal membrane. Sodium ions, which are the ionic species furthest from equilibrium across the cell membrane, flow down an electrochemical gradient into the neurones. This influx of positive charge is reflected in the

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1 The sustentacular cells retain their secretory function even after degeneration of the olfactory cells.

2 This accounts for the changes in impedance which have been monitored (Gesteland et al, 1965).
detection of a negative-going potential change by a recording electrode positioned in the mucus (Dodd and Squirrell, 1980; also see Gesteland, 1971).

The generator current (i.e. the flow of sodium ions), which passes down the neurones discharging the membrane potential, flows out of the cell in the region of the axon hillock; at this point, the cell membrane begins to respond to the depolarisation by generating an action potential which travels down the axon. In its wake, the resting potential of the membrane is restored by the efflux of potassium ions, such that a further flow of generator current is required before another impulse can be initiated. Thus, the magnitude of the generator current can control the frequency of neuronal discharge. The current flow from the axon hillock back to the mucus layer, which completes the circuit, must be via the supporting cells, since tight-junctional complexes (see Section 1.1(ii)) seal off the mucus layer from extra-cellular spaces within the epithelium (Dodd and Squirrell, 1980). The current flow through the epithelium is shown in Figure 1.20.

The EOG exhibits a dependency upon the transepithelial resistance\(^1\) which in turn is largely determined by that of the supporting cell membrane; this membrane has a higher resistance than that of the external milieu. Thus, the physical status of the supporting cell membrane will influence the magnitude of the EOG. The responses obtained to anaesthetic agents should be considered in the light of this dependence, since these reagents have been shown to cause changes in the physical properties of membranes (Seeman, 1972).

This knowledge of current flow in the olfactory epithelium has been partly obtained from studies which have measured the

\[ V_{\text{EOG}} = I_{\text{generator current}} \times R_{\text{transepithelial resistance}}; \]

see Thurm (1972).
Figure 1.20  Diagram showing the flow of generator current through the olfactory epithelium

Taken from Dodd and Squirrell (1980); based on Gesteland (1971)
variation in EOG peak amplitude, or rate of change of the EOG voltage, with depth in the epithelium (Figure 1.21; see Byzov and Flerova, 1964). The depth at which impulses are initiated in the newt epithelium has been determined by Shibuya and Tonosaki (1972); a maximum spike height was obtained at the depth of the neuronal cell bodies (150µ below the surface). This finding is consistent with previous studies on the frog epithelium (Ottoson, 1956) which indicated that the EOG response began to disappear at about this depth.

1.2(vi) Intensity coding

Neural coding for the intensity of an olfactory stimulus follows the general pattern found in other sensory modalities: the frequency of impulse generation in the olfactory neurones increases with odorant concentration (Dodd and Squirrel, 1980). The concentration ranges covered by individual neurones vary from 0.5 to 2.0 logarithmic steps (Mathews, 1972) whereas the sensitivity range of the epithelium as a whole, as monitored by the ECG response, has been found to cover 4.0 to 5.0 logarithmic steps (see Poynder, 1974a,b). This is because the neurones vary in sensitivity and, as the stimulus concentration is increased, units with lower sensitivities are recruited to contribute to the overall response.

The extreme sensitivity of the olfactory system is made possible by a high degree of neural convergence; the convergence ratio of olfactory neurones to secondary cells in the olfactory bulb has been reported to be about 10,000 : 1 in rabbits (Allison

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1 E.g. the odour threshold in water of 2-isobutyl-3-methoxypyrazine is 0.002 parts/10^9 (see Teranishi et al, 1974).

2 The olfactory system employs principles of information processing similar to those used in man-made pattern-recognition systems (see Uttal, 1973; Holden, 1976; Sampson, 1976; also see Fersaud and Dodd, 1982).
Figure 1.21 Variation in EOG peak height with depth in the epithelium

Taken from Ottoxon (1956)
and Warwick, 1949), bats (Bhatanger and Kallen, 1975) and turbots (Gemne and Døving, 1969). The significance of this convergence, which has been recognised for some time (Deutsch, 1967), has recently been discussed elsewhere (Holley and Døving, 1977; van Drongelen, 1978; van Drongelen et al, 1978; Kauer, 1980): a small increase in the firing rate of the peripheral neurones, which is undetectable at the single cell level, becomes a significant event at the bulbar level where all the responses are integrated. Such a mechanism functions optimally if the sensory neurones possess a low level of resting activity (van Drongelen et al, 1978); this has been found in single unit studies. Findings on the average rate of spontaneous discharge of frog olfactory neurones have included values of 3.5 ± 2.5 per minute (O'Connell and Kozell, 1969), 6 per minute (Daval et al, 1972) and 3 - 7 per minute¹ (Altner, 1974; also see Holley et al, 1974). "Silent units", which only fire in response to stimulation, have been widely reported, as have a smaller number of units with a higher spontaneous activity (e.g. 134 ± 25 spikes per minute; O'Connell and Kozell, 1969); these latter units may respond by having their activity suppressed.

Employing convergence to obtain sensitivity demands a large number of receptors. However, the individual receptors are not required to possess high sensitivity (Dodd and Squirrell, 1980).

¹ A stimulated cell will possess a firing rate of up to 1,200 spikes per minute (Revial et al, 1978a).
1.3 Theories of transduction and coding in the primary olfactory neurones

There are two outstanding biochemical problems involved in the olfactory process: the transduction mechanism, whereby the interaction of an odorant with the primary olfactory neurones initiates a receptor potential, and the quality coding mechanism which operates at the level of the primary neurones (Menevse et al., 1977b, 1978). To date, a variety of mechanisms have been proposed for these key steps, but most of them have received only limited experimental support (e.g. see Beidler, 1971; Fcynder, 1974c).

It is now accepted that the initial event in odour perception is the recognition of odorant molecules by receptors present in the olfactory mucosa (Pelosi et al., 1982). In general, much is known about receptor mechanisms (e.g. see Quatrecasas and Hollenberg, 1976; Houslay and Stanley, 1982) and about the structure of membranes (Chapman, 1968; Chapman and Wallach, 1973): the great variety of receptor systems appear to be dependent upon universal molecular mechanisms operating through a limited number of biopolymer types (Dodd and Persaud, 1981). Consequently, it seems likely that the olfactory system may not possess unique biochemical mechanisms. Indeed, by analogy with other receptor systems (Dodd, 1974; Greaves, 1976), transduction and coding are likely to be intrinsic properties of a macromolecular receptor complex" (Dodd and Persaud, 1981). Such speculations have led to the proposal of the allosteric membrane enzyme (AME) hypothesis for olfaction (Dodd et al., 1977; Dodd, 1978; also see Mooser, 1981); this is an example of a general hypothesis for the arrangement and function of receptor systems. The model takes into account the biochemical features of the olfactory system that have been previously implicated in alternative mechanisms, and conveniently considers all the postulated modes of action (Dodd and Persaud, 1981).
These general biochemical features, which may be discernible in olfactory cilia are diagrammatically represented in Figure 1.22.

1.3(i) The site of transduction

A common event in all chemoreceptor phenomena is the interaction of stimulus molecules (ligands) with receptors. The idea that olfactory cilia represent the loci of olfactory receptor sites has been postulated for many years (e.g. see Hopkins, 1926; Ottoson, 1956, 1970, 1971) but has proved somewhat controversial (see Cagan and Rhein, 1980; Rhein and Cagan, 1980, 1981): arguments against an essential role for cilia in olfaction have been presented (e.g. see Tucker, 1967; Moulton, 1976). Although most sense organs contain cilia or modified cilia\(^1\) (Rhein and Cagan, 1981), exceptions include the taste receptors of vertebrates, the eyes of many invertebrates and the vomeronasal organ, all of which contain microvilli (Cagan and Rhein, 1980; see Section 1.1(ii)j).

An early investigation into possible ciliary involvement was inconclusive (Shibuya, 1964; but see Ottoson, 1970, 1971) but subsequent studies have found that cilia are essential for odorant recognition (Shibuya and Tucker, 1967; Shibuya, 1969; Bronstein and Minor, 1977): the ECGs recorded to chemical stimuli were noted to decline following the removal of frog olfactory cilia with detergent, and to re-appear upon cilia regeneration\(^2\). More recently, direct biochemical evidence has been provided for the ciliary-binding of odorant molecules, supporting the hypothesis that odorant recognition sites are integral parts of the cilia (Rhein and Cagan, 1980, Cagan and

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\(^1\) E.g. modified cilia are the sensory organelles in retinal rod cells (Hagins, 1979), the inner ear (De Reuck and Knight, 1968) and protozoa (Browning et al, 1976).

\(^2\) The complete recovery of the ECG did not coincide with the complete regeneration of the cilia (Bronstein and Minor, 1977; also see DeSimone, 1981).
FIGURE 1.22  

SOME GENERAL BIOCHEMICAL MECHANISMS  
SUGGESTED IN OLFACTORY CILIA

This figure represents several general receptor mechanisms which have been suggested previously for the membrane interaction and recognition of various ligand molecules and which may be discernible in olfactory cilia. Small ligands such as typical odorants can interact with the lipid regions of cell membranes producing a variety of effects which could bring about the production of a receptor potential through conformational changes in ion gating proteins (see Section 1.3(ii)a). Further, the breakdown of phosphatidylinositol, which is provoked by muscarinic cholinergic stimulation, is involved in bringing about increased cell-surface permeability of Ca\(^{2+}\) which may function as a second messenger (see Section 1.3(i)). Finally, the (specific) interaction of ligands with membrane proteins may lead to activity changes of membrane-bound enzymes (e.g. Na\(^+\)-K\(^-\)-ATPase, adenylate cyclase) suggesting an involvement in the transduction process (see Section 1.3(ii)c).
Figure 1.22  Some general biochemical mechanisms suggested in olfactory cilia

Taken from Dodd and Persaud (1981)
Rhein, 1980).

Various anatomical studies (see Reese, 1965, Menco et al, 1978) have indirectly provided further backing for the involvement of cilia; ciliary intrusion into the mucus places these elements closest to the external environment (Dodd and Squirrel, 1980; also see Ottoson, 1970) and so it is reasonable to infer that the ciliary membrane represents the locus of odorant receptor sites.

Electrophysiologic studies on the frog have shown that the amplitude of the EOG response attains a maximum value when the recording microelectrode is positioned such that its tip contacts the surface of the mucus where cilia are also present (Ottoson, 1956). These cilia (which are morphologically distinct from rhythmically-beating cilia, thereby indicating possible specialisation) possess numerous granules on the membrane surface which have been suggested as possible receptor sites (Menco et al, 1976; Menco 1977a,b, 1978, 1980b; Kerjaschki and Horandner, 1976).

It has been concluded, from a theoretical standpoint, that olfactory cilia are capable of initiating and conducting effective electrical potentials to the dendrites provided that they are assumed to possess special properties, like those of olfactory nerve fibres\(^1\) (Ottoson and Shepherd, 1967). In addition, Atema (1973) has proposed a role for cilia microtubules in transducing odorant information (see Rhein and Cagan, 1981).

Although no data are yet available concerning the actual composition of ciliary membranes from air-breathing vertebrates (see Hormung and Mozell, 1981; Goldstein and Cagan, 1981), their overall structure may be imagined as an arrangement of proteins and phospho-

\(^1\) Certain objections to the notion that cilia initiate olfaction have also been discussed by Ottoson and Shepherd (1967).
lipids that is generally disposed towards the fluid-mosaic model of membranes (see Dodd and Persaud, 1981). It will be interesting to discover the amount of phosphatidylinositol in the sensory membrane, since this will determine the possibility of a transduction mechanism based on the breakdown of this phospholipid (Michell et al., 1976).

Evidence against the supporting cells functioning as the olfactory receptor sites has been provided by the observation that they do not respond to odorants (as opposed to anaesthetic compounds) when isolated in the degenerating epithelium (see Ottoson, 1956; Byzov and Flerova, 1964; Tucker and Shibuya, 1965; Takagi and Tajima, 1965). However, they may play a role in the electrical mechanism of signal formation (see Tucker and Shibuya, 1965).

1.3(ii) Theories of transduction

1.3(ii)a Penetration and puncturing: role of phospholipids

The first proposal for an olfactory transduction mechanism possessing a realistic theoretical basis and supported by experimentation involved membrane lipids (Dodd and Persaud, 1981); the so-called "Penetration and Puncturing" theory postulated that the diffusion of an odorant molecule through the membrane left behind it a hole which healed comparatively slowly thereby permitting the passage of ions such that membrane depolarisation occurred (Figure 1.23). The progressive breakdown of the cell wall was regarded as constituting the nerve impulse. This hypothesis, however, was formulated prior to molecular mechanisms of transduction in chemoreception have been reviewed by Kurihara et al. (1981).


Davies (1970, 1971) has described the "Penetration and Puncturing" theory as the modern counterpart of atoms "tearing their way into our senses and rending our bodies by their inroads" (Lucretius, 47 B.C.).
In a, an organic odorant molecule is adsorbed, penetrating a lipid region of the cell wall. In b, the odorant molecule is shown diffusing rapidly through the biomolecular lipid membrane, with a sharp hole being left behind it. Through this channel ions may then exchange, initiating the generator current which in turn releases the nervous impulse.

Taken from Davies (1971)
to the emergence of a detailed understanding of membranes, and so, despite being consistent with studies on the squid giant axon (Hodgkin and Katz, 1949) and on the anion permeability of the olfactory receptor membrane (Takagi et al, 1966), must be re-evaluated in the light of current knowledge.

It is now accepted that small ligands such as typical odorants do not puncture membranes to create transient holes. However, they can interact with the lipid regions of cell membranes to produce a variety of effects, such as phase transitions or altered packing of lipid molecules, which in turn may lead to membrane labilisation or stabilisation depending upon the odorant concentration and alteration of lipid-protein interactions (see Figure 1.24, Dodd and Persaud, 1981; also see Seeman and Weinstein, 1966, Dodd et al, 1970; Dodd and Squirrell, 1980; Kurihara et al, 1981). These actions could bring about the production of a receptor potential through conformational changes in ion-gating proteins. It has been shown that changes in the electrical conductance of highly purified phosphatidylcholine bilayers can be induced by odorants over the same concentration range as that to which the olfactory system responds (Cherry et al, 1970). Odorants can also increase the surface pressure of phospholipid monolayers in correlation with their thresholds (Koyama and Kurihara, 1972; also see Kurihara et al, 1981).

1.3(ii)b Vibration and olfactory pigment

The idea that the yellowy, olfactory pigment¹ might be able to absorb the "vibrations of odour", thereby affecting the contiguous cells in which the olfactory nerves end, was first speculated by Ogle (1870). More recent suggestions that the "triggering" of the nervous

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¹ For a general review of the olfactory pigment, see Moulton (1971).
Human erythrocytes were protected or stabilised against hypotonic and mechanical haemolysis in the presence of low concentrations of n-octylaldehyde, whereas high concentrations brought about increased lysis. The presence of the non-odorant saccharine exerted little effect on the amount of haemolysis observed.

Experimental conditions were as described by Seeman and Weinstein (1966).

Taken from Dodd and Persaud (1981)
impulse arises from molecular vibrations (Wright, 1954, 1964, 1966; Demerdache and Wright, 1967; reviewed in Wright and Burgess, 1970; also see Miles and Beck, 1949; Randebrock, 1966) have led to the postulate that "a particular vibration frequency in the molecule enables it to 'fire' one kind of receptor but not another, and the receptors use a frequency coding of a very different sort to distinguish their signals from those of receptors of other types" (Wright et al, 1967). Although each odour appeared to be associated with a distinctive major frequency component, various common components were noted in the responses to odours in the same stereochemical category. This finding, whilst seeming to provide neurophysiological evidence for the so-called stereochemical theory of olfaction (e.g. see Amoore, 1952, 1962a,b, 1963, 1964; also see Section 1.3(ii)c) (see Hughes and Hendrix, 1967) was cited as supporting the molecular vibration theory of olfaction (Wright et al, 1967; also see Wright, 1968, 1974). However, subsequent studies (see Davies, 1971) including observations on some enantiomeric pairs of chemicals1 have called into question the hypothesis relating odorous quality to patterns of low-frequency intra-molecular vibrations (for discussion, see Wright, 1978a; also see Wright, 1977, 1978b; Wright and Burgess, 1975; Hayward, 1977).

The discovery of carotenes in the olfactory regions of dogs and cows prompted Briggs and Duncan (1961, 1962) to suggest that such compounds play a dominant role in the primary events of olfactory transduction (also see Rosenberg et al, 1968). However, their apparent absence in rabbits, sheep, rats and pigs led to this proposal being questioned (see Koulton, 1962; Takagi and Yajima, 1965; Koulton and Beidler, 1967).

---

1 Some enantiomeric pairs possess recognisably different odours when perceived by some (but by no means all) human subjects (see Friedman and Miller, 1971; Leitereg et al, 1971a,b; Russell and Hills, 1971; Chloff, 1972; Lensky and Blum, 1974; also see Section 1.4(1)).
Enzymes and specific sites

The direct involvement of enzymes in olfactory perception (thereby implying specific "sites" for particular odours) has been contemplated for some time (see Davies, 1971). Kistiakowski (1950) claimed that odorant action was based upon the inhibition of one or more enzymes (related to a number of basic ("primary") odours) involved in the sensory nerves. Subsequently, it was shown that enzymes were localised in the nasal mucosa of the rabbit, with different enzymes occurring in different areas (Baradi and Bourne, 1951).

Biochemical studies suggesting Na\(^+\)-K\(^+\)-ATPase as a candidate enzyme system (Duncan, 1964; Koch 1969; Dodd, 1970) and indicating a possible enzyme-odorant interaction have been previously reported (Koch, 1971/1972, 1973; Koch and Desaiah, 1974; Koch and Gilliland, 1977; Rossi and Koch, 1981; also see Dreesen and Koch, 1982). This enzyme-perturbation hypothesis states that the stimulation and/or inhibition\(^1\) of the Na\(^+\)-K\(^+\)-dependent ATPase activity from nerve ending particles of olfactory bipolar sensing cells is in some way responsible for, or associated with, changes in the electrophysiological response (Koch and Gilliland, 1977; Rossi and Koch, 1981).

Studies utilising the EOG response of the sensory tissue have provided evidence for the specific involvement of cAMP in the olfactory transduction mechanism\(^2\) (Figure 1.25; Menevse, 1977;).

---

1 Antibodies to anisole binding protein (Price, 1978; also see Goldberg et al. 1979) have recently been reported to prevent the odorant stimulation of Na\(^+\)-K\(^+\)-ATPase activity from cow olfactory tissue (Koch et al., 1981).

2 Cyclic nucleotides fulfil a transduction role in several types of cells, including neurones (Braun and Birnbaumer, 1975; Greengard, 1976). For a discussion of their involvement in processes of photoreception, see Pober and Bitensky (1979) (also see Chapter 8).
Figure 1.25  
Peak amplitudes of EOGs to pentyl acetate from various preparations of olfactory epithelium after 2.5 min. exposure

![Graph showing peak amplitudes of EOGs to pentyl acetate from various preparations of olfactory epithelium after 2.5 min. exposure.](image)

Taken from Dodd and Persaud (1981)
Menevse et al, 1977a; also see Kurihara and Koyama, 1972; Minor and Sakina, 1973; Menevse et al, 1974; Dodd and Persaud, 1981). The observed effects were quite complex; the potent phosphodiesterase inhibitor, SQ 20,009\(^1\) was found to exert opposite effects on the epithelium obtained from frog and sheep. The sheep tissue did not respond to the inhibitor or to dibutyryl cAMP (Figure 1.25), but did respond to an increased level of cGMP with a transient increase in the amplitude of the ECG obtained (Squirrell, 1978).

Although not directly implicated in the transduction process, it is worthy of note that several other enzyme activities have recently been detected in olfactory epithelia (see Section 7.1(iii)).

Studies on the metabolism of androst-16-enes in porcine nasal epithelium have shown that the boar pheromone 5α-androst-16-en-3-one (see Section 6.1(iii)) is reduced by a cytoplasmic 3α-hydroxysteroid dehydrogenase\(^2\) to 5α-androst-16-en-3α-ol (an-α) and by a 3β-hydroxysteroid dehydrogenase\(^2\) to 5α-androst-16-en-3β-ol (an-β);

androsta-4,16-dien-3-one is converted in low yield to 5α-androst-16-en-3-one by a 4-en-5α-reductase (Gennings et al, 1974). Moreover, the 3α- and 3β-reduction of 5α-androstenone was found to occur to different extents in different nasal tissues; the predominant product in septum and respiratory tissues appeared to be an-α, whereas in right and left ethmoturbinate tissue, the ratio of an-α to an-β was more variable (Gower et al, 1981).

A variety of species have recently been reported to contain high concentrations of cytochrome P-450-dependent

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1. 1-ethyl-4-(isopropylidenehydrazine)-1H-pyrazole-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester.

2. The subcellular location, biochemical characteristics and possible significance of 3-hydroxysteroid dehydrogenases in porcine nasal tissue have been previously discussed by Gower et al (1981).
monooxygenases\textsuperscript{1} in the nasal cavity which may be important in
chemical-induced tumorigenesis (Hadley and Dahl, 1982; Dahl \textit{et al},
1982); in the dog and rat, the nasal carcinogen hexamethylphosphor-
amide was shown to be metabolised (N-demethylated) by nasal
microsomal enzymes to another known nasal carcinogen, formaldehyde
(Dahl \textit{et al}, 1982; Dahl and Hadley, 1983). Further investigations
"have indicated that, with some exceptions, the best substrates for
metabolism to formaldehyde\textsuperscript{2} by nasal microsomes tend to be those
containing N-methyl groups and which have some water solubility"
(Dahl and Hadley, 1983; also see Section 6.5). It has been suggested
that released formaldehyde may influence the irritancy of inhaled
compounds and contribute to the tumorigenicity of some substances
(Dahl and Hadley, 1983).

The steric theory of odour, which was reformulated in modern
terms by Moncrieff\textsuperscript{3} (1949, 1951, 1954), postulates that air-borne
molecules are "smelled when they fit into certain complementary
receptor sites on the olfactory nervous system" (Amoore, 1964);" if a chemical is volatile, and its molecules have the appropriate
configuration to fit closely into the receptor site, then a nervous
impulse will be initiated, possibly through a mechanism involving
disorientation and hence depolarization of the receptor cell membrane"
(Amoore, 1963). The identification of several specific anosmias and

\textsuperscript{1} These xenobiotic metabolising enzymes facilitate the removal of
lipid-soluble compounds by converting them to water-soluble
metabolites that may be excreted. However, metabolites of some
compounds are highly toxic (e.g. benzo[\textit{a}]-pyrene and the solvent
hexamethylphosphoramide) (Dahl \textit{et al}, 1982).

\textsuperscript{2} Formaldehyde, a product of hexamethylphosphoramide metabolism,
is also a frequent product of other cytochrome P-450-dependent
monooxygenase catalysed oxidations.

\textsuperscript{3} The idea goes back to Lucretius (47 B.C.)
primary odours (see Amoore, 1952, 1962a, b, 1963, 1964, 1967, 1969, 1970a, b, 1971; Amoore et al, 1964, 1967, 1969), together with various structure-activity studies (see Section 1.4(1)) have provided support for this hypothesis. Early work related seven "primary odours" (whose identification was based on frequency of occurrence) to the external shape of the odorant molecule; in addition precise site profiles and dimensions were proposed corresponding to the primaries (e.g. see Amoore, 1952, 1962a, b, 1964). However, a number of objections to this stereochemical theory were raised (for review, see Davies, 1971), and Amoore (1965; also see Amoore et al, 1967) eventually adopted a somewhat less rigorous approach.

More recently, the phenomenon of specific anosmia or "odour blindness" (c.f. colour blindness) has been used to identify primary odours (e.g. see Amoore, 1967); it is assumed that "the olfactory epithelium of an affected person lacks the specific receptor protein for detecting one whole family of odorants that belong to one of the primary odours" (Amoore, 1974; also see Amoore, 1977). The first primary odour to be elucidated in detail was the sweaty odour developed by isovaleric acid and its congeners (see Amoore, 1967); subsequently, primaries have been identified for the camphoraceous (1,8-cineole), fishy (trimethylamine), malty (isobutyraldehyde), minty (1-carvone), musky (1-pentadecalactone), spermous (1-pyrroline) and urinous (5a-androst-16-en-3-one) classes of odorants (see Amoore, 1969; Amoore and Forrester, 1976; Amoore et al, 1975, 1976, 1977; Pelosi and Viti, 1978; Pelosi and Pisanelli, 1981; also see Hendriks and Punter, 1980).

1 For comprehensive accounts of structure-activity relationships in chemoreception, see Benz (1976); Beets (1978).

2 Specific anosmia has also been reported to occur in mice (see Price, 1977; Wysocki et al, 1977; Wysocki, 1984).
Quality coding

The initial step in olfaction is generally thought to involve the adsorption of stimulus molecules onto receptor sites through a reversible binding interaction (Bests, 1971; Ottoson, 1971). However, in higher animals at least, odorant molecules do not have immediate access to such sites, but must first be drawn past the sorptive surfaces presented by a multichannelled flow path in the nasal cavity and then pass over the sorptive surfaces of antecedent mucosal regions before diffusing through the mucus to gain final access to the receptors themselves (Hornung and Mozell, 1981; also see DeSimone, 1981). These access barriers have been previously implicated in the initial stages of odorant discrimination (Moncrieff, 1967; Hornung and Mozell, 1981).

Two possible mechanisms which may underlie olfactory discrimination at the level of the olfactory mucosa have been proposed: the receptors may be selectively sensitive to different odorants, and/or the molecules of different odorants may spread differentially across the mucosa both in time and space (Adrian, 1950, 1953, 1954). It was reasoned (Adrian, 1950) that the observed spatiotemporal encoding of odorants in the olfactory bulb (Adrian, 1950; Mozell and Pfaffmann, 1954; Mozell, 1958; also see Moulton, 1965, 1967; Freeman, 1978) reflected analogous space-time activity patterns occurring at the mucosal level (also see Constanzo and Mozell, 1976; Moulton, 1976; Constanzo and O'Connell, 1978).

---

1. It should be emphasised that these two mechanisms need not be mutually exclusive, but could very well act in concert (Adrian, 1953).

2. The olfactory mucosa is topographically represented in the bulb (Le Gros Clark, 1957).

3. For discussions of some spatial characteristics of central information processing in the vertebrate olfactory pathway, see Holley and Devine (1977); Rauer (1980); also see Rauer et al (1984); Meredith (1984).
Support for a differential adsorption of odorants across the nasal epithelium (though not specifically across the olfactory epithelium) was first obtained with an in vitro sheep preparation (Moncrieff, 1955). Additional backing was provided by a brief theoretical discussion which pointed out that the molecules of some odorants may be bound less tightly to the olfactory receptors than those of other odorants, and so would travel faster and further across the mucosa (see Mozell and Jagodowicz, 1974). However, in order to maintain that differential sorption possesses any significance for olfactory discrimination, it was necessary to demonstrate at the mucosal level not only that molecules of different chemicals differentially migrate across it, but also that this difference is reflected in the neural discharges transmitted to the central nervous system: the findings of Mozell (1964a,b, 1966, 1967, 1969, 1970; for review see Mozell, 1971; but also see Hornung and Mozell, 1981) "may have provided" such demonstrations (Mozell and Jagodowicz, 1974). Moreover it was suggested that a "chromatographic-like" process might underly the separation of odorant molecules across the mucosal sheet (Mozell, 1970). A major objection though to this chromatographic model of olfactory discrimination asserted that the mucosas of most animals are "too short to allow adequate resolution in the separation of different odorants" (Mozell and Jagodowicz, 1974). Consequently, in an attempt to measure more directly how chemicals migrate across the olfactory mucosa, Mozell and Jagodowicz (1973)

1 Although considerable evidence supports the existence of spatial patterns (e.g., see Daval and Leveteau, 1969; Mustaparta, 1971; Daval et al, 1972; Kauer and Moulton, 1974; Koch and Desai, 1974; Moultan, 1976; Koch and Gilliker, 1977; Thomasen and Deving, 1977; Squirrell, 1978; Kubie et al, 1980), "few behavioural studies either support or refute these patterns as a mechanism for discrimination" (Hornung and Mozell, 1981; but see Dodd and Persaud, 1981).
substituted the \textit{in vivo} frog olfactory sac for the usual column of a gas chromatograph; their results (see Figure 1.26) indicated that in spite of the small size of the frog's olfactory mucosa, molecules of different chemicals do indeed migrate across it at very different rates (also see Mozell and Jagodowicz, 1974). Conclusive evidence for the existence of molecular distribution patterns was obtained with the bullfrog; by using tritium-labelled odorants (butanol, octane and butyl acetate), it was shown that a given compound establishes a particular distribution pattern along the intact olfactory sac, and that within a wide range of stimulus parameters (flow rates, concentrations, times) the distribution remains fairly constant (Hornung \textit{et al}, 1975).

The distribution of molecules of different odorants along the olfactory receptor 'sheet' appears to depend upon their partitioning between the air and mucosal phases; the more this partition favours the mucosa, the slower the molecules will travel (Hornung and Mozell, 1981). This mucosa/air partitioning has been previously measured using tritium-labelled odorants (Hornung \textit{et al}, 1979, 1980); for butanol, a water/air partition coefficient determined by the isotope technique was in good agreement with that determined by a more traditional gas flow technique (Amoore and Buttery, 1978). Calculations from diffusion coefficients and experimental measurements (Bostock, 1974) suggest that odorants will rapidly reach the surface of the epithelium\(^1\) (see Section 1.1(ii)c).

Research in olfactory electrophysiology has striven to discern some set of "discriminable electrophysiological responses"

\(^1\) For discussions of the accessibility of odorant molecules to olfactory receptors, see Laffort \textit{et al} (1974); Getchell and Getchell (1977); Hornung and Mozell (1981).
The drawing on the left shows the diagrammatic representation of the apparatus used by Mozell and Jagodowicz (1973) to measure odorant retention times across the olfactory mucosa of the bullfrog.

The graph on the right shows the relative retention times across the olfactory mucosa for the 15 odorants tested.

●, Highest partial pressure for each chemical at room temperature;
X, partial pressure ~ 0.56 mm Hg

Taken from Hornung and Mozell (1981)
for any set of "discriminable odorants" (Blank, 1974; also see Gesteland, 1971). However, the failure to discover such a simplistic mechanism has frustrated attempts to resolve a key issue in olfaction: the selective tuning of receptors to some physicochemical parameter of molecules (Blank, 1974). Several studies have illustrated the complexity of the neural code (e.g. see Gesteland et al., 1963; Shibuya and Shibuya, 1963; Mathews and Tucker, 1966; Mathews, 1972), leading Gesteland et al. (1965) to suggest that the stimulus-receptor response relationship was one of "utter chaos" (but see Lettvin and Gesteland, 1965). However, subsequent studies on the frog olfactory epithelium (O'Connell and Mozell, 1969) indicated that this relationship was not random (chaotic) even though different units did respond to the same odorants in more than one way. Additional examinations (O'Connell and Mozell, 1969; Mathews, 1972) have led to the suggestion that single receptors may possess more than one type of site for a particular odorant (Mathews, 1972; also see Gesteland, 1976). This concept of receptor site specificity (see Blank, 1974) has received experimental support from several independent lines of investigation, most notably from chemical modification studies (e.g. see Getchell, 1971; Getchell and Gesteland, 1972; Kenneve, 1977; Squirrel, 1978; Persaud, 1980; also see Sections 3.1(ii) and 5.1(i)) and from data on specific anosmics (e.g. see Amoore, 1969; also see Section 1.3(ii)c).

The recording of single unit activities to a battery of test odorants has provided an insight into odour discrimination by frog

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1 Studies on single turkey vulture receptors, which found that units differ from one another in their sensitivity to odorants, prompted the tentative suggestion that some units might signal intensity and some quality, whilst others might not contribute to sensation at all (Shibuya and Tucker, 1967).
olfactory receptors (see Figure 1.27; Duchamp et al, 1974; Revial et al, 1978a,b, 1982; also see Duchamp, 1982). These receptor cells were found to combine multiple sensitivities and consequently not to fall under simple categories; the degree of receptor specificity varied widely, with receptors responding to between 0% and 70% of the test odorants (Holley, 1974; also see Duchamp et al, 1974). In addition, an enhancement of specificity (by 29 - 100%) was shown to occur at lower odorant concentrations (Mathews, 1972).

Receptor (or "acceptor") sites are believed to be membrane proteins which exhibit low affinity and low specificity binding\(^1\) (Holley, 1974). Evidence for their proteinaceous nature has been provided by various chemical modification studies\(^2\) (e.g. see Getchell, 1971; Getchell and Gesteland, 1972; Menevse, 1977; Menevse et al, 1977b, 1978; Squirrell, 1978; Persaud, 1980; Shirley et al, 1980, 1981, 1983a; also see Sections 3.1(ii) and 5.1(i)).

In summary, the vertebrate olfactory system appears to have evolved in such a way that the needs for discrimination and sensitivity are reconciled in order to achieve a high degree of both features (Dodd and Squirrell, 1980).

---

\(^1\) Low specificity binding may account for the ability of the olfactory system to interact with virtually every volatile compound; it may also help in the removal of odorants (Dodd and Squirrell, 1980). Information processing (see Section 1.2(vi)) may compensate for the low sensitivity which is implied by low specificity.

\(^2\) The phenomenon of specific anosmia (see Section 1.3(ii)c) has also been cited as evidence for olfactory receptor proteins (Price, 1981).
Figure 1.27

Diagrammatic representation of unit responses to a battery of odorants

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Odorant</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEN</td>
<td>Benzene</td>
</tr>
<tr>
<td>ANI</td>
<td>Anisole</td>
</tr>
<tr>
<td>BRO</td>
<td>Bromobenzene</td>
</tr>
<tr>
<td>DIC</td>
<td>1,3-Dichlorobenzene</td>
</tr>
<tr>
<td>EHO</td>
<td>Thiophenol</td>
</tr>
<tr>
<td>ISA</td>
<td>Anisaldehyde</td>
</tr>
<tr>
<td>ANE</td>
<td>Anethole</td>
</tr>
<tr>
<td>BZO</td>
<td>Benzophenone</td>
</tr>
<tr>
<td>CIN</td>
<td>Cineole</td>
</tr>
<tr>
<td>CAM</td>
<td>dl-Camphor</td>
</tr>
<tr>
<td>TBU</td>
<td>Tert-butyl alcohol</td>
</tr>
<tr>
<td>XCN</td>
<td>Cyclohexanone</td>
</tr>
<tr>
<td>XCL</td>
<td>Cyclohexanol</td>
</tr>
<tr>
<td>ABU</td>
<td>n-Butyric acid</td>
</tr>
<tr>
<td>VAL</td>
<td>n-Valeric acid</td>
</tr>
<tr>
<td>IVA</td>
<td>iso-Valeric acid</td>
</tr>
<tr>
<td>CAP</td>
<td>Caproic acid</td>
</tr>
<tr>
<td>BCL</td>
<td>Butanethiol-1</td>
</tr>
<tr>
<td>SUL</td>
<td>Diethylsulphide</td>
</tr>
<tr>
<td>TIO</td>
<td>Thiophene</td>
</tr>
</tbody>
</table>
The area of the circles is approximately proportional to the spike frequencies (spikes min\(^{-1}\)) that were used in the quantitative evaluation of odour discrimination by frog olfactory receptors. Along a line a response appears as a larger spot (excitation) or a smaller spot (inhibition) with respect to the equally-sized spots representing the resting frequency of a nerve unit.

Taken from Revial et al (1978)
1.4 Biochemical aspects of odorants

1.4.1 Odorants as ligands

The ligands of many receptor systems possess easily discernible molecular features (see Greaves, 1976). However, there is no recognisable structural requirement for a molecule to function as an odorant, save that it be sufficiently volatile to reach the olfactory epithelium (Dodd and Persaud, 1981). The odorants shown in Table 1.3, which are particularly interesting for human olfaction, are all small ligands spanning less than an order of magnitude in molecular weight.

The type of odour possessed by a molecule is a subtle function of the molecular properties; the size and shape of the molecule, together with the distribution of polar groups, have been reported to determine the odour type (Amoore, 1970a; Dodd, 1976). However, the structural requirements for any particular odour type have only been partially defined (Boelens, 1974, 1976; Beets, 1978; Ohloff and Flament, 1979, MacLeod, 1980; also see Boelens et al, 1983). Some polar groups provide an odorant series with a distinctive odour type: the lower fatty acids possess a sweaty note (see Chapter 5); the lower thiols an easily recognisable type of putrid note, and the lower amines a fishy note. Although other common polar groups have a less definite odour impact, it is feasible for these particular odorant series to envisage the appropriate binding sites containing complementary polar groups (Dodd and Persaud, 1981).

Odour qualities themselves, and to a lesser extent taste qualities, are "extremely difficult to define in a numerical way that is appropriate for correlation with physicochemical parameters" (Schiffman, 1983). However, much promise in this respect is offered by the recently developed methodology of multidimensional scaling (MDS) (Schiffman et al, 1981); this powerful mathematical technique
### Table 1.3 Odour properties of typical odorants

<table>
<thead>
<tr>
<th>Molecular weight</th>
<th>Odorant</th>
<th>Odor type</th>
<th>Olfactory threshold&lt;sup&gt;b&lt;/sup&gt; (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>Methanethiol ( \text{CH}_3\text{SH} )</td>
<td>Putrid</td>
<td>0.02</td>
</tr>
<tr>
<td>59</td>
<td>Trimethylamine ( (\text{CH}_3)_3\text{N} )</td>
<td>Fishy</td>
<td>0.47</td>
</tr>
<tr>
<td>104</td>
<td>Methional</td>
<td>Boiled potatoes</td>
<td>0.2</td>
</tr>
<tr>
<td>132</td>
<td>Limonene</td>
<td>Lemons</td>
<td>10</td>
</tr>
<tr>
<td>166</td>
<td>2-isobutyl-3-methoxypyrazine</td>
<td>Green peppers</td>
<td>0.002</td>
</tr>
<tr>
<td>224</td>
<td>Cyclopentadecanone</td>
<td>Musk</td>
<td>15</td>
</tr>
<tr>
<td>272</td>
<td>5α-Androst-16-en-3-one</td>
<td>Urinous</td>
<td>0.18</td>
</tr>
</tbody>
</table>

<sup>a</sup> Odor description from Arctander (1969).

<sup>b</sup> Olfactory thresholds are for odorant solutions in water and are taken from the compilation given in Ohloff and Flament (1979).

Taken from Dodd and Ferseud (1981)
permits molecules to be arranged spatially using experimental measures of perceived flavour similarities as input (e.g. see Schiffman, 1974; Southwick and Schiffman, 1980). The odour quality space of several simple substituted pyrazines (see Section 1.4(ii)) with MDS of similarity judgements (see Figure 1.28) has been developed by Schiffman and Leffingwell (1981). No simple correlations were found between molecular weight, boiling point, molecular shape, water solubility, infrared spectra, ultraviolet absorption maxima, n.m.r. shifts for ring protons, refractive index or the three-dimensional odour quality space. However, computer-developed molecular descriptors did appear to possess some predictive value (see Schiffman, 1983). Such modest beginnings show that MDS of similarity judgements provides relevant flavour quality data to correlate with physicochemical variables. As the relevance of these parameters "becomes better determined, not only can computer design of flavour molecules become feasible, but also our understanding of the physiological determinants of flavour quality will advance" (Schiffman, 1983).

Odorants differ from the ligands of many other receptor systems in their stereochemical requirements for activity. For saturated monofunctional molecules, whose enantiomers show very little difference in odour quality and only slight differences in aroma intensity (e.g. menthol, isoborneol, octan-2-ol), there is only the possibility of a single type of interaction with the receptor site (Beets, 1978); the sole polar group can be used for "orienting the ligand at the binding site using molecular interactions such as hydrogen bonding and electrostatic interactions that have a directional element" (Dodd and Persaud, 1981). However, the addition of a second function (e.g. a double bond, an aromatic nucleus, or a
Figure 1.28 Two-dimensional cross sections through the three-dimensional space achieved by INDSCAL for pyrazines.

INDSCAL is one of several specially developed computer programmes used to analyse similarity judgements. This figure illustrates the odour quality spatial arrangement of several simple substituted pyrazines obtained with MDS of such judgements.

Taken from Schiffman (1983)
second functional group) might permit bifunctional interaction with a suitable bifunctional receptor site. Thus, it is feasible that "identical molecules could interact mono- or bifunctionally depending on their orientation, hence giving a difference in aroma response" (MacLeod, 1980). Although it is unclear whether such bifunctional olfactory interactions can explain the phenomenon of enantiomers with completely different aromas, the two most striking examples of this effect (nootkatone; see Haring et al., 1972: carvone; see Leitereg et al., 1971a) do possess structures capable of bifunctional interaction (also see Chloff and Giersch, 1980).

Studies of the molecular parameters responsible for the release of ambergris odour have lead to the proposal of a "triaxial rule of odour sensation": "at least three principal points of attachment at the receptor site can be described in terms of molecular features" (Chloff, 1980). The principle of this molecular rule has been shown to be capable of extension to certain types of natural substances, such as sesquiterpenes derived from the eudesmanes and eremophilanes.

The sensory evaluation of over 60 synthesised androstane and estrane derivatives has permitted molecular parameters to be established for the release of a "steroid-type" scent (Chloff et al., 1983). Odour perception with O-containing compounds in both classes was found to be regioselective. Osmophoric groups at C(3) were found to be the most active and specific, whilst functionality at C(2) was accompanied to a large extent by anosmic defects; O-containing substituents at C(1) and C(4) appeared to affect the receptor membrane in exceptional cases.

1 The terms "regioselective", "diastereoselective" or "enantioselective" indicate "substrate-receptor interactions in which one positional isomer, diastereoisomer or enantiomer leads to a different sensory response (in quality and/or intensity) than another" (Chloff et al., 1983).
A further characteristic of the "steroid-type" scent was **diastereoselectivity**: the odour intensity of axial 2- and 3-hydroxysteroids was far greater than that of the equatorial epimers, and epimeric hydroxy-groups in the 1-, 4- and 5- positions led to an almost complete absence of odour. Additionally, steroids were observed to follow the "triaxial rule of odour sensation", since only compounds with "normal" ring junctions and configuration were found to be odorants; steroids with cis-junctions between rings A and B or C and D were practically inactive.

A remarkable feature of the studies on steroid odorants was the finding of **enantioselectivity**, whereby C19- steroids of the "natural" enantiomeric series possessed extremely low (<6 ppb) perception thresholds whilst the corresponding "unnatural" enantiomers were found to be essentially odourless; it was noted that this appeared "to be the first reported instance of a total enantioselective response to an odorant" (Chloff et al, 1983).

Overall, no obvious correlation is evidenced between olfactory thresholds and molecular size (see Table 1.3). The olfactory threshold may reflect several molecular parameters, and with increasing molecular size, there may be "'compensating interactions' between these parameters analogous to enthalpy-entropy compensation found in the thermodynamics of some biological systems" (Dodd and Persaud, 1981).

### 1.4(ii) Pyrazine odorants

The work described in this thesis employed a variety of heterocyclic odorants, and in particular several compounds related to 1,4-diazine (pyrazine; Figure 1.29; also see Section 3.2 (iii)).

---

1 The terms "regioselective", "diastereoselective" or "enantioselective" indicate "substrate-receptor interactions in which one positional isomer, diastereoisomer or enantiomer leads to a different sensory response (in quality and/or intensity) than another" (Chloff et al, 1983).
Pyrazine (m.p. 54 - 56°C; b.p. 115 - 116°C), which is weakly basic (pK<sub>a</sub> 0.6), is a stable, colourless compound soluble in water.
The organic chemist has had a long association with pyrazine compounds (e.g. see Laurent, 1844; Schrotter, 1879, Wolff, 1888; Stoehr, 1893), although it was not until the mid 1960s that their occurrence in foodstuffs was widely noted. Since then, "pyrazines have been characterised as significantly contributing to the unique flavour and aroma associated with the roasting or toasting of numerous foods" (Calabretta, 1978). However, naturally-occurring pyrazines have also been isolated from food systems that have not undergone heat treatment (e.g. 27 raw vegetables; Murray and Whitfield, 1975), thus indicating that various biological pathways for pyrazine formation exist. To date, a vast array of pyrazine compounds have been reportedly identified in a wide variety of food systems (Table 1.4) (for reviews, see Maga and Sizer, 1973, 1975; Chloff and Flament, 1979; Vernin and Vernin, 1982).

Studies with model systems have yielded information on possible pathways of pyrazine formation (Figure 1.30; see Shibamoto and Bernhard, 1977, Calabretta, 1978; Shibamoto et al, 1979; for review see Maga and Sizer, 1973, 1975). Koehler et al (1969) found that in a low moisture model system pyrazines were formed via carbohydrate decomposition, followed by the interaction of these materials with nitrogen-containing molecules (i.e. amino acids). Fundamentally, this is the Maillard reaction ("Browning reaction") (see Calabretta, 1978; Vernin and Parkanyi, 1982). Thus, it would appear that in a food system where both sugars and amino acids are present, bound amino acid nitrogen is the primary contributor to the nitrogen found in the ring structure of pyrazines. It has also been shown that the primary carbon source for pyrazine formation comes from sugars (Koehler et al, 1969; also see Koehler and Odell, 1970; Shibamoto and Bernhard, 1977). Murray et al (1970) have postulated that the
<table>
<thead>
<tr>
<th>Foods from which pyrazines have been isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roasted peanuts</td>
</tr>
<tr>
<td>Roasted pecans</td>
</tr>
<tr>
<td>Roasted filberts</td>
</tr>
<tr>
<td>Pressure-cooked beef</td>
</tr>
<tr>
<td>Pressure-cooked pork liver</td>
</tr>
<tr>
<td>Fried lean veal meat</td>
</tr>
<tr>
<td>Boiled beef</td>
</tr>
<tr>
<td>Heated beef fat</td>
</tr>
<tr>
<td>Roasted beef drippings</td>
</tr>
<tr>
<td>Chicken broth</td>
</tr>
<tr>
<td>Popcorn</td>
</tr>
<tr>
<td>Potato chips</td>
</tr>
<tr>
<td>Explosion puffed dehydrated potatoes</td>
</tr>
<tr>
<td>Potato fusel oil</td>
</tr>
<tr>
<td>Molasses fusel oil</td>
</tr>
<tr>
<td>Beer</td>
</tr>
<tr>
<td>Jamaican rum</td>
</tr>
<tr>
<td>Scotch whisky</td>
</tr>
<tr>
<td>Deep fat-fried soybeans</td>
</tr>
<tr>
<td>Fermented soybeans</td>
</tr>
<tr>
<td>Hydrolysed soy protein</td>
</tr>
<tr>
<td>American white bread</td>
</tr>
<tr>
<td>Rye crispbread</td>
</tr>
<tr>
<td>Roasted barley</td>
</tr>
<tr>
<td>Cocoa products</td>
</tr>
<tr>
<td>Coffee products</td>
</tr>
<tr>
<td>Milk protein (casein)</td>
</tr>
<tr>
<td>Whey powder</td>
</tr>
<tr>
<td>Stale skim milk powder</td>
</tr>
<tr>
<td>Galbanum oil</td>
</tr>
<tr>
<td>Sesame oil</td>
</tr>
<tr>
<td>Green bell peppers</td>
</tr>
<tr>
<td>Green peas</td>
</tr>
<tr>
<td>Tomatoes</td>
</tr>
<tr>
<td>Dried mushrooms</td>
</tr>
<tr>
<td>Canned sweet corn</td>
</tr>
</tbody>
</table>
Figure 1.30  Summary of pyrazine formation pathways

Taken from Maga and Sizer (1975)
naturally-occurring (methoxy)pyrazines isolated from non-heated foods (e.g. see Murray and Whitfield, 1975) can be derived through the amidation and condensation of α-amino acids with α,β-dicarbonyls found in plant tissues with the resulting products being easily methylated. Microbial metabolism has also been cited as a means of pyrazine production¹ (see Kosuge and Kamiya, 1962; Demain et al, 1967; also see Reineccius et al, 1972).

Although it is generally agreed that pyrazines found in foods contribute to characteristic flavour, comparatively few reports have dealt specifically with the taste and odour properties of individual pyrazines. However, some characteristic odour descriptions have been noted together with data on the odour and/or taste thresholds of these compounds (Table 1.5; see Buttery et al, 1969; Seifert et al, 1970, 1972; Naga and Sizer, 1973, 1975; Parliment and Epstein, 1973; Pittet and Kruza, 1974; Takken et al, 1975; Calabretta, 1978).

Members of this odorant class have some of the lowest olfactory thresholds known. They are found in some naturally-occurring oils (e.g. galbanum oil, petitgrain oil, camphor oil (white), fenugreek extract, lovage root oil) and have become of great importance to the perfumery industry because of their interesting "green" odour (see Riezebos, 1972; Teranishi et al, 1974); several pyrazine derivatives have also been shown to function as alarm pheromones² utilized by hymenopterous genera (see Blum, 1974).

Studies on the odour thresholds in water of a series of methoxypyrazines related to 2-isobutyl-3-methoxypyrazine (see Chapter 6)

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¹ It should be noted that riboflavin constitutes a condensed pyrazine derivative; various pyrazines may be produced during its synthesis and degradation (Naga and Sizer, 1973).

² For a general review of pheromones, see Birch (1974).
<table>
<thead>
<tr>
<th>Compound</th>
<th>Odour description</th>
<th>Odour threshold (in parts of compound per $10^9$ parts of water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylpyrazine</td>
<td>Nutty, roasted</td>
<td>105,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60,000 b</td>
</tr>
<tr>
<td>2,3-Dimethylpyrazine</td>
<td>Green, nutty</td>
<td>2,500 c</td>
</tr>
<tr>
<td>2,5-Dimethylpyrazine</td>
<td>Earthy raw potato</td>
<td>35,000 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,800 d</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,000 e</td>
</tr>
<tr>
<td>Trimethylpyrazine</td>
<td>Nutty, roasted</td>
<td>9,000 b</td>
</tr>
<tr>
<td>Tetramethylpyrazine</td>
<td>Fermented soybeans</td>
<td>10,000 b</td>
</tr>
<tr>
<td>Ethylpyrazine</td>
<td>Nutty, roasted</td>
<td>22,000 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6,000 c</td>
</tr>
<tr>
<td>2-Ethyl-3-methylpyrazine</td>
<td>Nutty, roasted</td>
<td>130 c</td>
</tr>
<tr>
<td>2-Ethyl-3,5-dimethylpyrazine</td>
<td>Nutty, roasted</td>
<td>15,000 b</td>
</tr>
<tr>
<td>Methoxypyrazine</td>
<td>Not characteristic</td>
<td>700 d</td>
</tr>
<tr>
<td></td>
<td>Sweet, nutty</td>
<td></td>
</tr>
<tr>
<td>2-Methoxymethylpyrazine</td>
<td>Ethereal character</td>
<td>150 h</td>
</tr>
<tr>
<td>2-Methyl-3-methoxypyrazine</td>
<td>Roasted peanuts</td>
<td>4 d</td>
</tr>
<tr>
<td></td>
<td>Nutty, earthy</td>
<td></td>
</tr>
<tr>
<td>2-Methyl-5-methoxypyrazine</td>
<td>Green, vegetable</td>
<td>15 h</td>
</tr>
<tr>
<td></td>
<td>character</td>
<td></td>
</tr>
<tr>
<td>2-Ethyl-3-methoxypyrazine</td>
<td>Raw potato</td>
<td>0.4 d</td>
</tr>
<tr>
<td></td>
<td>Earthy, bell pepper</td>
<td></td>
</tr>
<tr>
<td>2-Propyl-3-methoxypyrazine</td>
<td>Bell pepper</td>
<td>0.006 d</td>
</tr>
<tr>
<td></td>
<td>d, i</td>
<td></td>
</tr>
<tr>
<td>2-Isopropyl-3-methoxypyrazine</td>
<td>Bell pepper, raw potato</td>
<td>0.002 d</td>
</tr>
<tr>
<td></td>
<td>Earthy, bell pepper</td>
<td></td>
</tr>
<tr>
<td>2-Isobutyl-3-methoxypyrazine</td>
<td>Bell pepper</td>
<td>0.002 d</td>
</tr>
<tr>
<td></td>
<td>d, i</td>
<td></td>
</tr>
<tr>
<td>2-Hexyl-3-methoxypyrazine</td>
<td>Bell pepper</td>
<td>0.001 d</td>
</tr>
<tr>
<td></td>
<td>d, i</td>
<td></td>
</tr>
<tr>
<td>2-Isobutyl-3-methoxy-5-methylpyrazine</td>
<td>Bell pepper, mint</td>
<td>0.3 d</td>
</tr>
<tr>
<td>Compound</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>2-Isobutyl-3-methoxy-6-methylpyrazine</td>
<td>Green pepper, <strong>d</strong></td>
<td>Pittet and Hruza (1974)</td>
</tr>
<tr>
<td></td>
<td>minty, camphoraceous</td>
<td>Koehler et al (1971)</td>
</tr>
<tr>
<td>2-Isobutyl-3-methoxy-5,6-dimethylpyrazine</td>
<td>Minty, camphoraceous <strong>d</strong></td>
<td>Guadagni et al (1971)</td>
</tr>
<tr>
<td>2-Isobutylpyrazine</td>
<td>Green, fruity <strong>a</strong></td>
<td>Deck and Chang (1965)</td>
</tr>
<tr>
<td>2-Isobutyl-3-methylpyrazine</td>
<td>Green (bell pepper like), <strong>k</strong></td>
<td>Buttery et al (1969)</td>
</tr>
<tr>
<td></td>
<td>dry and sweet notes</td>
<td>Takken et al (1975)</td>
</tr>
</tbody>
</table>

**Notes:**
- **d** indicates a difference in perception.
- **a** indicates a specific type of green note.
- **k** indicates a specific type of green note.
isolated from green peppers showed that the addition of methyl
groups to the ring increased the odour threshold by a factor of
about $10^5$. The threshold was also increased by a factor of about
$10^5$ when the methoxy group was removed (Figure 1.31; Teranishi et
al, 1974; see Chapter 7). However, increasing the chain length of
the 2-alkyl-3-methoxypyrazines greatly reduced the odour threshold
(see Figure 1.32). In this series, the odour of the hexyl and
propyl compounds was similar to bell peppers, whilst the ethyl com-
 pound possessed a raw potato aroma and the methyl compound had an
odour similar to that of peanut butter (Buttery et al, 1969; Seifert
(Figure 1.33; Teranishi et al, 1974) showed that increasing the length
of the side chain progressively decreased the odour threshold. How-
ever, unlike the methoxypyrazines, the introduction of additional
groups onto the pyrazine ring decreased, rather than increased, the
odour threshold. It is worthy of note that 2-ethyl-3,6-dimethyl-
pyrazine exhibited the most potato-like odour of this series of
pyrazines; its threshold was "essentially the same" (Teranishi et al,
1974) as 2-ethyl-3-methoxypyrazine which also possessed a characteristic
raw potato odour. In general, however, the thresholds of the methoxy-
pyrazines were noted to be much lower than those of the alkylpyrazines,
thereby indicating an important function of the methoxy group with
respect to odour threshold (Deck and Chang, 1965; Mason et al, 1966;
Bondarovich et al, 1967; Rizzi, 1967; Buttery et al, 1971; Guadagni
et al, 1971, 1972; see Chapter 7). More recent studies on a variety
of substituted pyrazines (Calabretta, 1978) have substantiated an
earlier investigation (Seifert et al, 1972) which noted that the most
potent odours were formed when an alkyl substituent occupied the ortho
position to a methoxyl function.
Figure 1.31  Structures and thresholds of methoxypyrazines

<table>
<thead>
<tr>
<th>Structure</th>
<th>Parts/10^9</th>
<th>Structure</th>
<th>Parts/10^9</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Structure" /></td>
<td>0.002</td>
<td><img src="image2" alt="Structure" /></td>
<td>0.001</td>
</tr>
<tr>
<td><img src="image3" alt="Structure" /></td>
<td>0.26</td>
<td><img src="image4" alt="Structure" /></td>
<td>0.006</td>
</tr>
<tr>
<td><img src="image5" alt="Structure" /></td>
<td>2.6</td>
<td><img src="image6" alt="Structure" /></td>
<td>0.425</td>
</tr>
<tr>
<td><img src="image7" alt="Structure" /></td>
<td>315</td>
<td><img src="image8" alt="Structure" /></td>
<td>4</td>
</tr>
<tr>
<td><img src="image9" alt="Structure" /></td>
<td>400</td>
<td><img src="image10" alt="Structure" /></td>
<td>700</td>
</tr>
</tbody>
</table>

Taken from Teranishi et al (1974)

Figure 1.32  Variation of olfactory threshold with chain length of 2-alkyl-3-methoxypyrazines

![Graph](image11)

Logarithm of olfactory threshold

- 2-methyl-3-methoxypyrazine
- 2-ethyl-3-methoxypyrazine
- 2-propyl-3-methoxypyrazine
- 2-isobutyl-3-methoxypyrazine
- 2-hexyl-3-methoxypyrazine

Number of carbon atoms in alkyl side chain
Figure 1.33  Structures and thresholds of alkylpyrazines

<table>
<thead>
<tr>
<th>Structure</th>
<th>Parts/10⁰</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure 1" /></td>
<td>175,000</td>
</tr>
<tr>
<td><img src="image2.png" alt="Structure 2" /></td>
<td>60,000</td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure 3" /></td>
<td>6,000</td>
</tr>
<tr>
<td><img src="image4.png" alt="Structure 4" /></td>
<td>400</td>
</tr>
<tr>
<td><img src="image5.png" alt="Structure 5" /></td>
<td>100</td>
</tr>
<tr>
<td><img src="image6.png" alt="Structure 6" /></td>
<td>20</td>
</tr>
<tr>
<td><img src="image7.png" alt="Structure 7" /></td>
<td>6</td>
</tr>
<tr>
<td><img src="image8.png" alt="Structure 8" /></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Structure</th>
<th>Parts/10⁰</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image9.png" alt="Structure 9" /></td>
<td>2500</td>
</tr>
<tr>
<td><img src="image10.png" alt="Structure 10" /></td>
<td>1800</td>
</tr>
<tr>
<td><img src="image11.png" alt="Structure 11" /></td>
<td>1500</td>
</tr>
<tr>
<td><img src="image12.png" alt="Structure 12" /></td>
<td>130</td>
</tr>
<tr>
<td><img src="image13.png" alt="Structure 13" /></td>
<td>35</td>
</tr>
<tr>
<td><img src="image14.png" alt="Structure 14" /></td>
<td>0.4</td>
</tr>
</tbody>
</table>

Taken from Teranishi et al (1974)
The possession of bell pepper-like odours has also been found for a number of very potent alkylthiazoles\(^1\) and, more recently, for five synthesised 4,5-dialkylthiazoles, whose odour thresholds were measured (see Figure 1.34; Bittery et al, 1976); the most potent, 4-butyl-5-propylthiazole (see Section 6.4(xi)), had an odour threshold of 3 parts per \(10^{12}\) parts of water\(^2\). These 4,5-dialkylthiazoles "differ from previous bell pepper-like aroma compounds in that they have no methoxy group" (Bittery et al, 1976).

\(^1\) For investigations into the relationship of bell pepper character and odour potency to chemical structure, see Seifert et al (1972); Parliment and Epstein (1973); Pittet and Kruza (1974).

\(^2\) 2-Isobutyl-3-methoxypyrazine has an odour threshold of 2 parts per \(10^{12}\) parts of water (Bittery et al, 1969).
Figure 1.34: Odour thresholds of some 4,5-dialkylthiazoles with potent bell pepper-like aromas, and of some related compounds.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>THRESHOLD PARTS PER $10^{12}$ PARTS WATER</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Structure I" /></td>
<td>1</td>
</tr>
<tr>
<td><img src="image2" alt="Structure II" /></td>
<td>10</td>
</tr>
<tr>
<td><img src="image3" alt="Structure III" /></td>
<td>20</td>
</tr>
<tr>
<td><img src="image4" alt="Structure IV" /></td>
<td>60</td>
</tr>
<tr>
<td><img src="image5" alt="Structure V" /></td>
<td>120</td>
</tr>
<tr>
<td><img src="image6" alt="Structure VI" /></td>
<td>190</td>
</tr>
<tr>
<td><img src="image7" alt="Structure VII" /></td>
<td>5,100</td>
</tr>
<tr>
<td><img src="image8" alt="Structure VIII" /></td>
<td>470,000</td>
</tr>
</tbody>
</table>

Related compounds:

Taken from Buttery et al (1976)
CHAPTER 2: GENERAL METHODS AND MATERIALS

2.1 Choice of experimental animals

Studies on mammalian olfactory mechanisms ideally require an animal preparation which is amenable to biochemical, behavioural and genetic examination (Shirley et al., 1983a). Previous electrophysiological investigations have employed in vivo frog preparations and in vitro sheep preparations (e.g. see Getchell, 1971; Getchell and Gesteland, 1972; Menevse, 1977; Squirrell, 1978; but also see Gesteland and Sigwart, 1977); these were also used in the course of this present work. In addition, an in vitro rat preparation was developed and characterised.

Each of these animal preparations displayed certain favourable qualities. Frogs were cheap, readily available and generally convenient. In addition, the studies performed previously on such preparations provided a useful reference point for future work. Consequently, most of the preliminary electrophysiological investigations were carried out on the frog. However, this in vivo preparation proved slightly unreliable: differences in the age, state of health and general welfare of the frogs, together with problems encountered during anaesthetism and subsequent dissection, caused such preparations to be somewhat variable in performance.

A relatively large amount of olfactory epithelium could be readily isolated from sheep (about 1 - 1.5g wet weight/animal) thus favouring their employment in biochemical studies of olfaction (e.g. ligand binding studies; see Chapter 6). However, in vitro sheep preparations also demonstrated variable electrophysiological behaviour; some preparations gave good EOG recordings almost immediately, others did so after a recovery period of up to four hours, whilst some simply
did not respond at all. In view of this irregularity, only a few exploratory studies were performed on such preparations; for the sake of brevity, these experiments are not reported in this thesis. Similarly, details of the tissue preparation and the recording of EOG responses from sheep are not presented: the appropriate methodology has been previously described (Squirrell, 1978). No significant differences were noted between the results obtained from sheep and those acquired from either the in vivo frog preparation or from the in vitro rat preparation.

Although frogs have been shown to respond behaviourally to odorants (Müller and Kiepenhever, 1976), as have other amphibians such as *Xenopus* (Kramer, 1933) and *Triton* (Matthes, 1927), the laboratory rat appears to be more suited to such investigations (see Shirley et al., 1983a). Thus, in an attempt to provide a link between behavioural, genetic and biochemical studies, an in vitro rat preparation was developed, and subsequently characterised, for electrophysiological work. This preparation exhibited the advantageous features of both the frog and the sheep whilst overcoming many of the problems associated with these animals. Consequently, most of the electrophysiological studies described in this thesis were performed on in vitro preparations of the rat.

2.2 Animal and tissue preparation

2.2(i) Preparation of frogs for in vivo electrophysiological recordings

In vivo recordings were performed on the common frog, *Rana temporaria* and occasionally on *Rana pipiens*; no differences were observed between the results obtained from the two species. Following delivery, the animals (male and female; about 20g each) were housed
for a period of days in a cold room\(^1\) (4°c); each frog was warmed and kept at room temperature for at least two hours (and preferably longer, e.g. overnight) prior to experimentation.

The frogs were anaesthetised by partial immersion in an aqueous solution of urethane\(^2\) (10\% (w/v); later studies employed 5\% (w/v)) until the reflex responses to eye poking and skin tweaking were abolished, whereupon further absorption of the anaesthetic was arrested by immediately rinsing the frog's skin with tap water. The frogs remained anaesthetised by this treatment for the entire course of the experiments and they were not allowed to recover.

The frog was covered with a moist tissue and rigidly fixed by "ear screws" to a perspex frame. Under a dissecting microscope, the olfactory eminence on one side of the head (the left hand side; Chapter 3, or the right hand side; Chapter 5) was exposed by cutting away the skin, cartilage and finally the epithelium of the roof of the nasal cavity (dorsal olfactory epithelium); great care was taken to prevent any spillage of blood on to the eminence. The perspex holder was then screwed into position beneath the adjustable odour applicator and electrode holders. A good blood circulation was found to be essential for obtaining stable EOG recordings over a period of hours.

For the electrophysiological studies involving concanavalin A (Chapter 5), the dissected frog was removed from the perspex holder and the buccal aperture of the internal naris was sealed with a small Teflon plug. The frog was then rigidly clamped into a water-cooled

---

1 For the electrophysiological studies described in Chapter 5, the frogs were maintained exclusively at room temperature prior to experimentation.

2 Ethyl carbamate.
perspex chamber (thus maintaining the preparation at 15 - 18°C)
attached to the olfactometer turntable, which in turn was screwed
into position such that the exposed eminence was correctly aligned
beneath the adjustable odour applicator (see Section 2.4).

2.2(11) Preparation of rats for in vitro electrophysiological recordings

Male Wistar rats\(^1\) (about 150g each), which were allowed food
and water ad libitum prior to the experiment, were killed by
cervical dislocation. The head was removed and cut longitudinally
to one side of the mid-line; normally, the dissection was such that
the olfactory apparatus contained in the right hand side of the head
remained intact. Next, the nasal septum and associated tissue were
removed to expose the olfactory turbinates. This entire section of
dissected head was then rigidly clamped into the glass fibre/epoxy
resin holder of the olfactometer turntable\(^2\), which in turn was
screwed into position beneath the adjustable odour applicator.

Preliminary studies on this in vitro preparation suggested
that the quality and stability of the EOG signals were influenced by
the speed of dissection (personal observations). Consequently,
superfusion of the olfactory turbinates with Ringer solution (see
Section 2.3) was performed as soon as practicable (usually ≤ 1 min)
after death.

2.3 Maintenance of rat olfactory epithelium in vitro

The olfactometer turntable was equipped with several stainless

\(^1\) No special precautions were taken to ensure pathogen-free animals.

\(^2\) For the electrophysiological studies involving concanavalin A
(Chapter 5), the turntable was water-cooled such that the
temperature of the in vitro preparation was maintained at 15 - 18°C,
thereby increasing its longevity. However, the vapour-phase
labelling experiments (Chapters 3 and 4) employed equipment which
was not so cooled. Consequently, the rat preparations used in
these studies remained at ambient temperature.
steel tubes which could be freely manoeuvred to provide inlet or outlet devices for various solutions.

At the outset of each experiment, the exposed olfactory turbinates (see Section 2.2(i)) were superfused with aerated Ringer solution\(^1\) for about five minutes. The solution was removed by an aspiration tube adjusted such that the epithelium was adequately bathed without any of the superfusing liquid spilling on to the turntable. Aeration was performed in a glass column (20 cm x 5 cm) with 95% O\(_2\)/5% CO\(_2\), which was introduced through a scinttered glass inlet; the solution was led off from the bottom of this column to the tissue preparation at 1 - 2 ml/min via PTFE tubing. All glassware and equipment was regularly cleansed in order to prevent microbial growth. The Ringer solution was kept in the equipment for only short periods (i.e. 1 - 2 days); it was stored in concentrated form at 4\(^\circ\)C.

A constant stream of deodorised, humidified air was played over the surface of the olfactory epithelium between odour pulses; this arrangement maintained the tissue in an active condition for several hours (see Section 4.3(i)).

The olfactometer turntable (Figure 2.1), which was made out of brass, was purpose-built\(^2\) for in vitro EOG recording. The elevated (about 15 cm above the baseplate of the odour applicator) table (incorporating the glass fibre/epoxy resin holder for the sectioned head; two ventilation ports for clean, humidified air (see Section 2.4(ii)a); the mounts for the recording and reference

---

\(^1\) For the composition of mammalian and frog's (see Section 5.2) Ringer solutions, see Dawson et al (1972).

\(^2\) Designed and constructed by Dr. S. G. Shirley, Department of Chemistry and Molecular Sciences, University of Warwick.
This photograph presents a general view of the rat olfactory preparation. The dissected head (1) is securely clamped in position on the glass fibre/epoxy resin holder (5) by the clear plastic sheet. The gap between the tissue and the plastic is sealed with silicone grease, thus creating a water-tight chamber over the olfactory turbinates. The two ventilation ports used for delivering clean, humidified air to the sectioned head are visible at the top of the photograph. The reservoir of the earth electrode (3), and the stainless steel tubes used for solution addition (1, 2) and removal (4) are shown as they are positioned during the recording of EOG signals.
Figure 2.1 The olfactometer turntable used for studies on the in vitro rat preparation.
electrodes, and the stainless steel tubes for solution addition/removal) was freely manoeuvrable in the horizontal plane, thus aiding the alignment of the olfactory turbinates with the odour applicator delivery tubes (see Section 2.4(ii)b); the brass plate could also be tilted (back and forth) to facilitate adequate superfusion of the olfactory turbinates with Ringer solution. The glass fibre/epoxy resin holder ensured that the sagitally-sectioned head remained electrically isolated from the surface of the turntable.

2.4 EOG recording and stimulus control

The EOG responses obtained from sheep, frogs and rats throughout the course of this present study involved the use of three different odour applicators; the vapour-phase labelling experiments on the in vivo frog preparation (see Chapter 3) employed equipment with which much previous work had been performed (e.g. see Menevse, 1977; Squirrell, 1978), whereas the labelling studies on the rat (see Chapters 3 and 4) and the investigations involving concanavalin A (see Chapter 5) utilised two new purpose-built applicators\(^1\) of similar and improved design.

This section describes the apparatus\(^2\) (i.e. olfactometer and applicators) used for the in vitro labelling studies (see Chapters 3 and 4) and for the examinations with Con A (see Chapter 5); the older set-up has been fully detailed elsewhere (e.g. see Menevse, 1977; Squirrell, 1978; Menevse et al, 1978; also see Bostock and Poynder, 1972; Poynder, 1974a,b).

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1 Designed and constructed by Dr. S. G. Shirley, Department of Chemistry and Molecular Sciences, University of Warwick.

2 For more detailed descriptions, see Shirley (1986).
2.4(i) EOG recording

Recording microelectrodes were pulled from silica glass tubing (1 mm i.d.) to give a tip diameter of 30 - 50 microns, and were filled with Ringer solution (gelled with 0.3% agarose); connection was made through a chlorided silver wire. The electrodes, each of which had a resistance of about 0.5 - 4.0 MΩ, were re-usable for several months; they were stored at room temperature, with the tips submerged in Ringer solution.

For EOG recording, the tip of the measuring electrode was placed just in contact with the surface of the olfactory mucus; the delivery tube of the odour applicator was stationed about 4 mm away and was directed straight at the point of contact. The spatial positioning of the microelectrode was varied according to the type of preparation being examined: with frogs, the tip was situated at the peak of the olfactory eminence¹, whereas with the in vitro rat preparations, it was placed over the third olfactory turbinate¹. In all cases, a micromanipulator was employed to manoeuvre the electrode which, once positioned, was rigidly held in place.

The earth (i.e. reference) electrode also comprised a chlorided silver wire. For the in vivo recordings, it was wrapped in Ringer-soaked muslin and placed in the frog's mouth. The in vitro studies on the rat employed a cylindrically coiled wire which was housed within a small plastic reservoir containing Ringer solution (gelled with 0.3% agarose); a curved strip of tissue-plugged silica

¹ Since previous studies have indicated that different regions of the receptor epithelium may be particularly sensitive to different odours (e.g. see Daval and Leveteau, 1969; Mustaparta, 1971; Daval et al., 1972; Kauer and Moulton, 1974; Squirrell, 1978; Kubie et al., 1980; see Section 1.2(i)), all of the electrophysiological investigations described in this thesis employed an experimental set-up which standardised the position of the recording microelectrode.
glass tubing (also filled with the gelled Ringer solution) connected this reservoir to the in vitro preparation. A good contact between the tissue plug and the sectioned head was necessary in order to obtain stable EOG responses.

Apart from the earth and recording electrodes, the preparations were electrically isolated. The signals were lead to a high impedance amplifier, via a pre-amplifier mounted on the micro-manipulator, and were recorded on a Servoscribe chart recorder.

2.4(ii) Stimulus control

The electrophysiological investigations described in this thesis required well-defined odorous stimuli.

2.4(ii)a Olfactometry

The present studies utilised an olfactometer (30 cm high x 60 cm long x 16 cm wide) previously constructed (see Menevse, 1977; Squirrell, 1978; Menevse et al, 1978) from the design of Poynder (1974a,b) (also see Bostock and Poynder, 1972; Bostock, 1971). Details of the odour generation system are shown in Figure 2.2.

Compressed air (>35 p.s.i.) was filtered, dried and deodorised before being split into two streams. One of these was further split into six (Chapters 3 and 4) or eight (Chapter 5) channels, which were independently controlled by pressure regulators and flow restrictors; each of these streams was passed (1 - 10 ml/min) over a pool of liquid odorant contained within a specially-made glass U-tube such that turbulent flow was achieved. The second stream of clean, dry air was

---

1 The tissue plug was normally placed in contact with the rat's tongue.

2 Standard solutions of odorants were prepared in either deodorised liquid paraffin or diethyl phthalate.

3 Deodorisation was achieved by passing the air stream through charcoal.
Figure 2.2  Schematic diagram of the odour generation system

A  Pressure regulator

B  Pressure gauge or manometer

C  Stainless steel tower containing silica gel, molecular sieve 4A, molecular sieve 13X, and activated charcoal

D  Humidifier

E  Centrifugal filter

F  Manifold

G  Capillary flow restrictor

H  U-tube containing odorant

I  Mixing chamber

J  Shut-off tap

K  Odorised air streams which are delivered to the odour applicator via PTFE tubing

L  Clean air streams

The diagram shows the method of connection without (1) and with (2,3) dilution. These methods are used as necessary to obtain the required final odour concentration.
humidified by steam injection and centrifugally filtered (to remove water aerosol and any condensation) before being split into seven (Chapters 3 and 4) or nine (Chapter 5) (carrier) channels; one of these air flows was delivered (1 litre/min) to the olfactory tissue between odour pulses\(^1\), whilst each of the remaining streams (regulated to flow at 250 ml/min) was mixed with one of the odorised air channels at the odour applicator (see Section 2.4(ii)b).

The odorant surface in the U-tubes presented to the air streams was 1 cm x 8 cm. The tubes themselves, which were housed in a thermostated water bath at sub-ambient temperature (usually 15\(^\circ\)C) in order to maintain steady odorant vapour pressures and to avoid odorant condensation in the downstream tubing, were cleaned in chromic acid, washed in double distilled water and ethanol, and baked until odorless; the PTFE tubing connecting the U-tubes to the odour applicator was replaced when the odorant to be carried was changed.

2.4(ii)b Stimulus switching

The in vitro vapour-phase labelling studies (Chapters 3 and 4) and the investigations involving concanavalin A (Chapter 5) utilised two purpose-built odour applicators of similar design and operation (see Figure 2.3). They consisted of seven (Chapters 3 and 4) or nine (Chapter 5) stainless steel tubes (2.8 cm internal diameter) which could be sequentially moved parallel to their axes by means of a mechanical system; one of these tubes presented moist, clean air (1 litre/min) to the tissue preparation between odour pulses, whilst the others delivered clean, humidified air which had been odorised.

---
\(^1\) The moist clean air was delivered to the in vitro rat preparation via one of the stainless steel tubes of the odour applicator (see Section 2.4(ii)b) and through a pair of adjustable vents situated on the olfactometer turntable adjacent to the sectioned head (see Figure 2.1).
This photograph presents a general view of the odour applicator. Odorous air streams are carried to the delivery system in PTFE tubing (4) and after being mixed with clean, humidified air are presented to the tissue preparation via stainless steel odour tubes (1), which can be moved in and out of the body of the applicator (pneumatically controlled; 3). When not being applied to the olfactory epithelium, odorised air flows are extracted from the applicator via a vacuum line (2). The stand (7) permits the height of the applicator and the delivery angle of the odour stream to be varied. Any external odours are removed from the area of the applicator via a vacuum line (6). The flame ionisation detector (8) used for stimulus monitoring is shown in position at the left-hand side of the photograph.
Figure 2.3 The odour delivery system
as previously described (see Section 2.4(ii)a).

During an odour stimulus, the mechanical system (pneumatically controlled) retracted the clean air tube into the body of the applicator and replaced it with an odour tube which was positioned about 4 mm from the surface of the turbinate (see Figure 2.1); the other odorised air-flows (and the clean, humidified air) were extracted from the applicator via a vacuum line, incorporating a trap. The mechanical system ensured not only accurate positioning of the odour tube but also very sharp switching of the odour stream, since adsorption of the odorant to the tubing of the delivery system was always at equilibrium. The profile of the odorant pulse, as determined by a modified flame ionisation detector situated near the surface of the mucosa, was rectangular within the time scale of the experiment. The movement of the tubes did not affect the tissue nor elicit an electrical response. The stainless steel tubes were thoroughly flushed with clean air before changing the odorised air stream.

2.4(ii)c Stimulus monitoring

The characteristics (i.e. wave-form and concentration) of the applied odorous stimuli were monitored routinely with either a gas sensor or a modified flame ionisation detector (F.I.D.) (see Bostock and Poynder, 1972); these devices have been employed in previous

1 Odorised air was similarly extracted when clean, moist air played on the olfactory tissue between odour pulses.

2 The forward movement of the tubes, which is driven by a pneumatic cylinder and piston, is very rapid (55 mm in about 30 msec.; mean acceleration in excess of 10xg; final velocity in excess of 200 cm/sec.); "while the nozzle is moving, it outruns the odour stream which is slowed on contact with the surrounding air" (Shirley, 1986).

3 TGS 812 gas sensor, obtained from Watford Electronics Ltd., 33 Cardiff Road, Watford, Hertfordshire.
studies of similar design (e.g. see Kenevse, 1977; Kenevse et al., 1978; Squirrell, 1978; Persaud, 1980).

The F.I.D. (see Appendix 1) was positioned between the delivery tube of the odour applicator and the tissue preparation (see Figure 2.3); it was calibrated absolutely for each odorant under investigation, whilst the day-to-day calibration was based upon the saturated vapour of the standard odorant isoamyl acetate. It should be noted that the odorant concentrations in the mucus near the receptors will differ from the concentrations monitored by the F.I.D., since the odorants possess different air/water partition coefficients (see Gesteland, 1984). In general, vapour-phase odorant concentrations (expressed as moles of odorant/litre of air (see Laffort, 1963)) giving EOG responses within the 1 - 10 mV range were employed (see Table 2.1).

2.4(ii) d Stimulus quality

Odorant purity was examined by gas-liquid chromatography using a Perkin-Elmer F11 chromatograph with a 20% DEGS Chromasorb W-HP (100 - 120 mesh) column (1.6 mm (outer diameter) x 1.8 m (length)). Nitrogen was employed as the carrier gas.

For the vapour-phase labelling experiments and ligand binding studies described in Chapters 3, 4 and 6, the odorants1 used were analysed as 99.5% pure, unless otherwise stated. Elsewhere, 98% purity was regarded as the minimum value acceptable (see Squirrell, 1978).

1 The characterisation of the radiolabelled odorant $[^3H]2$-isobutyl-3-methoxyprazine is discussed in Section 6.2.
<table>
<thead>
<tr>
<th>Odorant</th>
<th>Vapour-phase concentration ($\mu M$)</th>
<th>Odorant</th>
<th>Vapour-phase concentration ($\mu M$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetic acid</td>
<td>0.32</td>
<td>phenacyl bromide</td>
<td>0.35; 0.18; 0.10</td>
</tr>
<tr>
<td>acetophenone</td>
<td>0.33</td>
<td>n-octanethiol</td>
<td>0.07</td>
</tr>
<tr>
<td>2-acetyl-3-methylpyrazine</td>
<td>0.27</td>
<td>phenacyl chloride</td>
<td>0.41</td>
</tr>
<tr>
<td>2-acetylpyrazine</td>
<td>0.19</td>
<td>c-pentane-carboxylic acid</td>
<td>0.36</td>
</tr>
<tr>
<td>3-acetylpyridine</td>
<td>0.32</td>
<td>n-propanol</td>
<td>0.23</td>
</tr>
<tr>
<td>acrylic acid</td>
<td>0.29</td>
<td>propiophenone</td>
<td>0.36</td>
</tr>
<tr>
<td>i-amyl acetate</td>
<td>0.93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-bromoacetyl-3-methylpyrazine</td>
<td>0.28</td>
<td>2-pyrazine-carboxylic acid</td>
<td>0.31</td>
</tr>
<tr>
<td>2-bromoacetylpyrazine</td>
<td>0.21</td>
<td>3-pyridine-methanol</td>
<td>0.29</td>
</tr>
<tr>
<td>3-bromoacetylpyridine</td>
<td>0.30</td>
<td>2-pyridinemethanol bromoacetate</td>
<td>0.36</td>
</tr>
<tr>
<td>t-butanethiol</td>
<td>0.05</td>
<td>3-pyridinemethanol bromoacetate</td>
<td>0.38</td>
</tr>
<tr>
<td>n-butyric acid</td>
<td>0.34</td>
<td>2-pyridinemethanol propionate</td>
<td>0.38</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>0.28</td>
<td>3-pyridinemethanol propionate</td>
<td>0.35</td>
</tr>
<tr>
<td>m-cresol</td>
<td>0.28</td>
<td>3-pyridylacetic acid</td>
<td>0.61</td>
</tr>
<tr>
<td>dimethylethylpyrazine</td>
<td>0.06</td>
<td>3-pyridylacetonitrile</td>
<td>0.74</td>
</tr>
<tr>
<td>3,5-dimethylbutyl acetophenone</td>
<td>0.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-dodecylpropionate</td>
<td>0.44</td>
<td>trimethylamine</td>
<td>0.18</td>
</tr>
<tr>
<td>ethyl 2-pyrazine-carboxylate</td>
<td>0.27</td>
<td>i-valeric acid</td>
<td>0.46</td>
</tr>
<tr>
<td>2-isobutyl-3-methoxypyrazine</td>
<td>0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-isopropyl-3-methoxypyrazine</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nicotinic acid</td>
<td>0.53</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This table indicates the vapour-phase odour concentrations applied to the *in vivo* frog preparation and/or the *in vitro* rat preparation throughout the course of the present studies.
2.5 Synthesis and derivatisation of odorants

The investigations described in this thesis necessitated the synthesis of several odorants. Product identification and purity were determined by a variety of analytical techniques, such as gas-liquid chromatography, nuclear magnetic resonance spectrometry, infrared and mass spectrometry, and by thin layer chromatography (see Section 6.2(ii)b), all compounds were judged to be at least 99.5% pure.

2.5(i) Synthesis of 2-isobutyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine

The synthesis of two 2-alkyl-3-methoxypyrazines (see Figure 2.4) was achieved by condensing the appropriate amino acid amide with glyoxal (Jones, 1949) to form the corresponding 2-alkyl-3-hydroxypyrazine which was converted to the methoxy derivative by methylation with diazomethane (see Seifert et al, 1970).

The amides of leucine and valine were prepared by converting the appropriate amino acid ester hydrochloride to the free ester by treatment with triethylamine (Chambers and Carpenter, 1955) prior to reaction with methanolic ammonia. The resulting amide (re-crystallised from a methanol/re-distilled diethyl ether solution) was reacted with glyoxal to form the appropriate 2-alkyl-3-hydroxypyrazine. At the end of the condensation process, the reaction mixture was neutralised (with 12 M hydrochloric acid) to produce a milky solution which was then concentrated to dryness under vacuum. The white solid residue was extracted with boiling chloroform to give crude crystalline product.

Diazomethane (in re-distilled diethyl ether) was added to an

---

1 The methylation of the 2-alkyl-3-hydroxypyrazines was performed by the technical staff of the Department of Chemistry and Molecular Sciences, University of Warwick.
Figure 2.1 Synthesis of 2-alkyl-3-methoxypyrazines

\[ \text{H}_2\text{NCHRCO}_2\text{CH}_3\cdot\text{HCl} \]

amino acid ester hydrochloride

\[ \quad \xrightarrow{\text{triethylamine}} \]

\[ \text{H}_2\text{NCHRCO}_2\text{CH}_3 \]

amino acid ester

\[ \quad \xrightarrow{\text{methanol/ammonia}} \]

\[ \text{H}_2\text{N-C=O} \quad \text{H}_2\text{N-CHR} \]

amino acid amide

\[ \quad \xrightarrow{\text{glyoxal}} \]

\[ \text{2-alkyl-3-hydroxypyrazine} \]

\[ \quad \xrightarrow{\text{diazomethane}} \]

\[ \text{2-alkyl-3-methoxypyrazine} \]
ice-cooled solution of the 2-alkyl-3-methoxypyrazine (in re-distilled diethyl ether), and following reaction at 5°C and 25°C, the excess diazomethane and ether were removed on a steam bath to leave the crude product which was distilled under diminished pressure to yield the purified odorant (see Table 2.2).

2.5(ii) Synthesis of ethyl 2-pyrazinecarboxylate

Ethyl 2-pyrazinecarboxylate was prepared by refluxing the appropriate acid (2-pyrazine carboxylic acid) with absolute ethanol (see Vogel, 1978). After cooling, the reaction mixture was slowly poured, with stirring, onto crushed ice and the resulting solution was rendered alkaline by the addition of ammonia solution. The product was extracted with re-distilled diethyl ether, and the combined ethereal extracts were dried over magnesium sulphate. The ether was removed by flash distillation, and the residue distilled under reduced pressure.

2.5(iii) Synthesis of 2- and 3-pyridinemethanol propionate

The propionate esters of 2- and 3-pyridinemethanol were prepared by refluxing the alcohols with propionic acid (Vogel, 1978). The reaction mixture was poured into water contained in a separating funnel, and the product was extracted with carbon tetrachloride. After washing with concentrated sodium hydrogen carbonate solution, the impure ester was dried over magnesium sulphate; product purification was achieved by adsorption chromatography on silica gel (60 - 120 mesh) using re-distilled diethyl ether as eluant.

2.5(iv) Synthesis of 2- and 3-pyridinemethanol bromoacetate

The synthesis of 2- and 3-pyridinemethanol bromoacetate was effected by the careful dropwise addition of bromoacetic acid (in re-distilled diethyl ether) to the appropriate alcohol (see Vogel, 1978). The reaction mixture was refluxed, and after cooling, the
Table 2.2  Spectral data of 2-isopropyl-3-methoxypyrazine and 2-isobutyl-3-methoxypyrazine

<table>
<thead>
<tr>
<th></th>
<th>2-isopropyl-3-methoxypyrazine</th>
<th>2-isobutyl-3-methoxypyrazine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass spectra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(recorded on a Kratos MS 60 mass spectrometer)</td>
<td>molecular ion 152</td>
<td>molecular ion 166</td>
</tr>
<tr>
<td></td>
<td>major ions 137, 124, 105, 95, 68, 54, 53, 52, 43, 41</td>
<td>major ions 165, 151, 125, 124, 109, 95, 94, 81, 68, 53, 41</td>
</tr>
<tr>
<td>Infrared absorption spectra</td>
<td>S (6.5, 6.8, 6.9, 7.1, 7.2, 7.5, 8.6, 8.9, 9.9)</td>
<td>S (6.5, 6.8, 6.9, 7.2, 7.6, 8.6, 9.9)</td>
</tr>
<tr>
<td>(recorded on a Perkin-Elmer 457 Grating Infrared Spectrophotometer)</td>
<td>M (7.7, 7.9, 8.4, 9.1, 9.2, 11.9)</td>
<td>M (7.4, 8.4, 9.4, 11.8)</td>
</tr>
<tr>
<td>NMR spectra (recorded on a Perkin-Elmer R3A NMR spectrometer)</td>
<td>8.78 (D, -CH-(CH₃)₂)</td>
<td>9.13 (D, -CH-(CH₃)₂)</td>
</tr>
<tr>
<td>(D₂O solvent; resonance frequencies in p.p.m. (T) downfield from TSS. S: singlet; M: doublet; M: multiplet)</td>
<td>6.60 (M, -CH-(CH₃)₂)</td>
<td>7.85 (M, -CH-(CH₃)₂)</td>
</tr>
<tr>
<td></td>
<td>6.05 (S, -CH₂)</td>
<td>7.33 (D, -CH₂-CH₂)</td>
</tr>
<tr>
<td></td>
<td>2.03 (M, ring H)</td>
<td>6.04 (S, -CH₂CH₃)</td>
</tr>
<tr>
<td></td>
<td>2.0 (M, ring H)</td>
<td>12.4 min.</td>
</tr>
<tr>
<td></td>
<td>retention time</td>
<td>23 min.</td>
</tr>
</tbody>
</table>

These spectral data are consistent with those presented by Seifert et al (1970) and by Maga and Sizer (1975)
resulting white crystals of product were washed and subsequently re-crystallised from re-distilled diethyl ether/dried methanol (1 : 9).

2.5(v) Bromination of acetophenone, 3-acetylpyridine, 2-acetylpyrazine and 2-acetyl-3-methylpyrazine

The synthesis of phenacyl bromide, 3-bromoacetylpyridine, 2-bromoacetylpyrazine and 2-bromoacetyl-3-methylpyrazine was performed as reported elsewhere (Cowper and Davidson, 1939); bromine was gradually added to the appropriate non-brominated starting material (dissolved in anhydrous re-distilled diethyl ether), after which the solvent and dissolved hydrogen bromide were quickly removed under reduced pressure. The resulting crystals of crude product were filtered, washed and re-crystallised from methanol.

2.6 Materials

All of the chemicals employed were of the highest purity available and were used without further purification unless otherwise stated. Solutions containing reagents were prepared in doubly distilled water immediately prior to use; where necessary, their pH was adjusted by the addition of dilute hydrochloric acid or sodium hydroxide. 2

Unless indicated otherwise below, solvents (AnalaR grade), inorganic salts and buffer materials were supplied by BDH Chemicals Ltd., Poole, Dorset, and odorants were obtained either from the collection of the Warwick Olfaction Research Group or from the Aldrich Chemical Company Ltd., Gillingham, Dorset.

1 Commercially-available phenacyl bromide (found to be about 70% pure) was employed in some experiments, after first being re-crystallised from methanol and analysed as >99.5% pure. Commercially-available phenacyl chloride was similarly treated prior to use.

2 For the NMR studies described in Chapter 7, odorant solutions were prepared in deuterium oxide and adjusted with deuterium chloride or sodium deuteroxide as required, unless otherwise stated.
The following materials were obtained from the sources stated:

Activated charcoal (untreated powder, 250-350 mesh), adenosine deaminase (type I, from calf intestinal mucosa), bovine serum albumin (fraction V powder, 98-99% albumin; essentially fatty acid free), butyl PBD, concanavalin A, EDTA, Folin and Ciocalteau's phenol reagent, HEPES, lauryl sulphate (sodium salt), myoglobin (type II, from sperm whale skeletal muscle), penicillin G (sodium salt) and Sigmacote were purchased from the Sigma Chemical Company Ltd., Poole, Dorset; bromoacetic acid, Diazald (precursor to diazomethane), diethyl phthalate, glyoxal, L-leucine methyl ester hydrochloride, 2-pyridinemethanol, 3-pyridinemethanol, urethane and L-valine methyl ester hydrochloride were supplied by the Aldrich Chemical Company Ltd., Gillingham, Dorset, and 1,8-cineole, m-cresol, isovaleric acid (sodium salt) (all purified by re-crystallisation) and isoamyl acetate were obtained from BDH Chemicals Ltd., Poole, Dorset.

Citronellol and diacetyl (both purified by chromatography on silica gel) were supplied by Bush Boake Allen Ltd., London, and Fisons Ltd., Loughborough, Leicestershire, respectively; pyridoxal 5-phosphate monohydrate was purchased from The Boehringer Corporation (London) Ltd., Lewes, East Sussex, and Blue Dextran 2000 and Sephacryl S200 Superfine were obtained from Pharmacia (Great Britain) Ltd., Hounslow, Middlesex.

\[ ^3\text{H} \] 2-Isobutyl-3-methoxypyrazine and 4-butyl-5-propylthiazole were generously donated by Dr. P. Pelosi, University of Pisa, Italy.

Plastic-backed t.l.c. plates (0.2 mm thick precoated, silica gel 60F) were purchased from Merek E., West Germany.

Male Wistar rats were obtained from Olac 1976 Ltd., Bicester,
Oxfordshire; frogs (*Rana temporaria*; *Rana pipiens*) from Gerrard Biological Supply, East Preston, West Sussex, and sheep's heads from Snelsons, Kenilworth, Warwickshire.
Preface to

CHAPTERS 3, 4 and 5: CHEMICAL MODIFICATION APPROACH TO THE Olfactory Code

It is widely believed that the initial step in olfaction concerns the interaction of odorants with the sensory membranes of the primary neurones. These neurones, in vertebrates, appear to be generalist cells which respond in a differential manner to a wide range of odorants (see Polak, 1973; Poynder, 1974c; Gesteland, 1976; Beets, 1978; Cagan and Mare, 1981). This has suggested that there are several types of olfactory receptor proteins which are distributed in differing relative concentrations amongst the primary neurones. Thus, one primary neurone may contain several types of receptor in its plasma membrane. On the basis of this model, the problem of the olfactory code can be reduced to the identification of the number of independent receptor proteins, together with the estimation of the possible types of interactions between them (Menevse et al., 1977b; Shirley et al., 1983a).

The chemical modification of ligand-binding sites is a classical approach to such identification (Menevse et al., 1977b; Dodd and Persaud, 1981), and it has been previously used with a variety of systems, including membranes (Barratt et al., 1969; Tinberg and Packer, 1976), enzymes (Shaw, 1970; Brocklehurst et al., 1970), bacterial chemoreceptors (Barber, 1976; Barber et al., 1979), protozoan chemoreceptors (Doughty and Dodd, 1978), invertebrate olfactory receptors (Villet, 1974; Frazier and Haytz, 1975) and vertebrate olfactory receptors (Getchell, 1971; Getchell and Gesteland, 1972; Menevse, 1977; Menevse et al., 1977b, 1978; Squirrell, 1978; Persaud, 1980; Criswell et al., 1980; Delaleu and Holley, 1980; Schafer et al., 1980; Shirley et al., 1980, 1981, 1983a).

The following chapters describe various studies that were performed on the in vivo frog and in vitro rat preparations. Chapters 3 and 4 detail
a series of vapour-phase modification experiments in which the reactive (i.e. brominated) derivatives of several homo- and hetero-cyclic odorants were employed as vapour-phase labelling reagents. Chapter 5 outlines the effect on the olfactory mucosa of treatment with the plant lectin, concanavalin A.
3.1 Introduction

3.1(i) Affinity labelling

In general, affinity labelling reagents (RL) possess two functionally-distinct parts: a biologically active moiety (R) capable of reversible complex formation with a given macromolecule (usually protein, P), and a correctly positioned, chemically reactive leaving group (L) (Wilchek and Bayer, 1978). The reagent interacts with its receptor to form initially a reversible protein-ligand complex (P . . . . R-L) and then a stable covalent bond with amino acid residues located at or near the protein active site (P - R + L\(^-\); see Figure 3.1) (see Zisapel and Sokolovsky, 1977). The formation of the reversible complex increases the local concentration of the reagent at the active site relative to its concentration in free solution; this favours covalent bond formation in the site, as compared to the rest of the macromolecule (Wofsy et al., 1962). A kinetic description of the affinity labelling process has been previously reported (Ketzer et al., 1963). The occurrence of an affinity labelling event may be established by several experimental criteria; such labelling should result in concomitant inactivation of the reversible binding activity, with unrelated protein being insignificantly labelled. Furthermore, the covalently-bound ligand should preferably be localised at the active site of the protein (Wilchek and Bayer, 1978). Finally, true affinity labelling should permit the demonstration of protection, whereby the specific ligand provides a certain amount of

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1 For review, see Singer (1970); Jakoby and Wilchek (1977).
2 For a general review of protein chemical modification, see Singer (1967); also see Shaw (1970); Thomas (1974).
Figure 3.1  Schematic representation of affinity labelling

\[ P + R - L \xrightleftharpoons[k_2]{k_1} P \cdots R - L \xrightarrow{k_3} P - R + L^- \]

\( P = \) receptor macromolecule

\( RL = \) affinity labelling reagent, comprising a biologically active moiety (R) and a chemically reactive leaving group (L)

\( P \cdots R - L = \) reversible receptor-ligand complex

\( P - R = \) covalent receptor-ligand complex

Taken from Wilchek and Bayer (1978)
protection against the affinity label (Singer, 1970). The factors relevant to the selection of an appropriate affinity labelling reagent have been summarised elsewhere (Wilchek and Bayer, 1978).

The identification of receptors in preparations of subcellular fractions is only feasible if they have been labelled prior to membrane disruption. In principle, affinity labelling affords the high degree of specificity needed for this purpose \(^1\) (Zisapel and Sokolovsky, 1977); the labelling of olfactory receptor sites in the intact system can be monitored indirectly through physiological parameters, such as the EOG\(^2\) or behavioural responses (e.g. see Singer et al., 1973).

3.1(ii) Chemical modification approach to the identification of olfactory receptors

One of the established methods for identifying ligand-binding sites on proteins involves their irreversible labelling with reactive chemicals (Dodd and Persaud, 1981); a large number of ligand sites in a wide range of biopolymers and membranes have been so investigated (Katzenellenbogen, 1977).

Structure-activity relationships from human olfactory studies have suggested that there may be receptor proteins for at least the urinous, musky, floral, fruity, minty and camphoraceous classes of odorants (Beets, 1978). Their existence, and that of other receptor proteins, could be verified if it were possible to selectively block the activity of each of these sites in the olfactory epithelium.

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1 It should be noted that although the utilisation of affinity labelling as a means of tagging receptors for identification, quantitation and isolation, is potentially of great value, the attempted selective labelling of such receptors also causes other structures in the crude state or intact cells to be concomitantly labelled, thereby increasing the background noise (Quatrecasas and Hollenberg, 1976).

2 The EOG responses obtained in the frog to various substances have been reportedly used to calculate dissociation constants which in turn serve as an index for the affinity between odorant and receptor site (Senf et al., 1980).
whilst retaining the activity of the other sites. Blocking would involve the covalent labelling of one or more amino acid side chains at the odorant binding site (Dodd and Persaud, 1981). The interpretation of such studies would depend upon the binding properties of the proteins under investigation. If well-defined odorants were to bind with a high degree of specificity and affinity to a single class of receptor protein, then it should be possible to inactivate this class of protein without affecting the activity of other classes of olfactory receptors. Conversely, if particular odorants were to bind to several classes of receptor proteins, then differential inhibition of binding activity may result.

Three distinct types of chemical modification methods have been applied to the identification of receptor proteins in the vertebrate olfactory system\(^1\). This section describes previous attempts that have been made to bring about the putative affinity labelling and photoaffinity labelling of such receptors; group-specific chemical modification is discussed in Chapter 5 (see Section 5.1(i)).

3.1(ii)a Affinity labelling

The identification of olfactory receptors by the use of putative affinity labelling odorants\(^2\) (see Section 3.4) has been attempted in the frog (e.g. see Squirrell, 1978) and in the rat (e.g. see Persaud, 1980). Utilising the bromoacetate group as an analogue for the propionate group (Figure 3.2), a series of potentially useful labelling reagents directed at fruity, minty,

\(^1\) The factors common to all three types have been previously noted (Dodd and Persaud, 1981).

\(^2\) Since the labelling reagents employed in these types of experiments are also odorants, they may be referred to accurately as labelling odorants.
Figure 3.2 Generalised structure of a putative affinity labelling reagent

\[ \text{BrCH}_2\text{C}_2\text{O} \]

The bromoacetate group is used as an analogue for the propionate group:

\[ \text{CH}_3\text{CH}_2\text{C}_2\text{O} \]

Van der Waal's Radii (in A°)

<table>
<thead>
<tr>
<th>Element</th>
<th>Radius (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>1.35</td>
</tr>
<tr>
<td>Cl</td>
<td>1.80</td>
</tr>
<tr>
<td>Br</td>
<td>1.95</td>
</tr>
<tr>
<td>I</td>
<td>2.15</td>
</tr>
<tr>
<td>-CH(_3)</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Ed. by R. C. Weast.
CRC Press, Inc., Cleveland, Ohio, U.S.A.
floral and woody classes of odorant binding sites have been synthesised by varying the size and shape of the R' group. The synthesis of reactive odorants for many other classes of receptor appears equally feasible (Dodd and Persaud, 1981).

A comprehensive vapour-phase labelling study of the fruity-odorant binding site in the frog olfactory epithelium has been performed by Squirrell (1978). The labelling reagent, ethyl bromoacetate, which gave a good EOG signal, preferentially inhibited its own response. Differential inhibition was obtained to other odorants; fruity odorants were affected to a greater extent than either minty or camphoraceous odours (Figure 3.3; also see Criswell et al, 1980). Such differentiality may provide a means of identifying different types of receptor sites. Similar findings of differential EOG inhibition have also been obtained from studies on the in vitro rat preparation which attempted to specifically label woody odorant (cyclododecyl propionate) receptor sites (see Persaud, 1980; also see Section 4.3(iv)).

3.1(ii)b Photoaffinity labelling

A disadvantage both of group-specific (see Chapter 5) and of affinity labelling is that the chemically reactive group in the labelling reagent confers irritant properties upon the compound. Thus, such odorants probably stimulate trigeminal receptors as well as olfactory receptors (Menevse et al, 1977b). Photoaffinity labelling\(^1\), however, overcomes this disadvantage by photolytically generating the appropriate reactive moiety at the binding site. This technique provides an extremely powerful method for the specific

\(^1\) For a general review of photoaffinity labelling, see Bayley and Knowles (1977).
Differential labelling with the reactive odorant ethyl bromoacetate

<table>
<thead>
<tr>
<th>Odorant</th>
<th>Odor</th>
<th>Inhibition of EOG between 60-90-sec labeling (%) (mean ± SD) (3-5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,8-Cineole</td>
<td>Camphoraceous</td>
<td>23.1 ± 8.7</td>
</tr>
<tr>
<td>tert-Butyl propionate</td>
<td>Minty</td>
<td>27.4 ± 8.0</td>
</tr>
<tr>
<td>Ethyl pivalate</td>
<td>Minty</td>
<td>34.8 ± 11.6</td>
</tr>
<tr>
<td>Ethyl propionate</td>
<td>Fruity</td>
<td>49.7 ± 3.9</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>Fruity</td>
<td>51.1 ± 10.5</td>
</tr>
<tr>
<td>Ethyl bromoacetate</td>
<td>Fruity and irritant</td>
<td>72.3 ± 6.5</td>
</tr>
</tbody>
</table>

Taken from Squirrell (1979)
labelling of receptor sites, as exemplified by studies on the chemoreceptors of the bacterium Pseudomonas aeruginosa (Barber et al., 1979). Photoaffinity labelling of the frog olfactory epithelium has been reported using a range of aromatic azido compounds (Menevse et al., 1977b; Delaleu and Holley, 1983); all of these molecules, which gave good EOG responses, are chemically unreactive in the dark, but decompose to reactive short-lived intermediates upon ultraviolet irradiation. Following illumination of the frog olfactory mucosa during constant stimulation with 1-azidonaphthalene vapour, a preferential inactivation of the receptors for this odorant was observed. The extent of inactivation varied between 60 - 100% of the original EOG value, depending upon the experimental conditions. Again (see Figure 3.3), the EOGs obtained to various other odorants were differentially inhibited (Figure 3.4). The chemical modification of the sensory membrane brought about by phenylazide was found to affect the responses "in relative ratios which could be predicted from a classification of the odorants based upon independent studies of receptor cell odour sensitivity" (Delaleu and Holley, 1983; also see Revial et al., 1978a,b): the responses to chemicals representative of the "aromatic group" (e.g. acetophenone, anisole, benzene, nitrobenzene and phenylazide) were more reduced than those obtained to compounds of the "camphoraceous group" (e.g. DL-camphor and 1,8-cineole).

This chapter describes the vapour-phase labelling of frog and ret olfactory receptor sites directed against various homo- and heterocyclic odorants (i.e. benzenoids, pyridines and pyrazines). The validity of the in vitro studies is discussed elsewhere in this thesis (see Chapter 4).
The effect of light on the EOG responses elicited by 1-azidonaphthalene in different parts of the frog olfactory mucosa.

Vertical arrows indicate illumination of the tissue at 326 nm. The EOGs obtained to 1-azidonaphthalene were preferentially inhibited (60 - 100% of the original value) following illumination of the mucosa during constant stimulation with this vapour. In control experiments, under the same irradiation conditions, the responses to naphthalene did not change by more than 10% of the original value.

Taken from Dodd and Fersaud (1981)
3.2 Methods

3.2(i) Experimental protocol: in vivo frog preparation and in vitro rat preparation

Vapour-phase labelling studies were performed on in vivo frog and in vitro rat preparations maintained at ambient temperature (i.e. 22 ± 2°C). The general methodology was independent of the tissue preparation used; for every experiment, several (at least 3) reproducible (i.e. variation ≤1 2%) EOG responses were obtained to each of the odorants under examination (apart from the vapour-phase labelling odorant). The peak amplitudes of these responses were then averaged to give the mean "100% value" (i.e. baseline response) for each compound against which subsequent electrophysiological responses could be compared. Only preparations giving stable EOGs during this baseline value determination were employed further. The time required to achieve EOG stability varied with different preparations; with the in vitro rat preparation, such responses were routinely obtained 15 - 20 minutes after the death of the animal.

Next, the applicator channel carrying the labelling odorant was switched on for a set period of time (usually twenty seconds), and the peak height of the resulting EOG was taken as the "100% value" for this odorant.

Following a rest period (recovery time) of approximately five minutes, at least duplicate EOG responses were obtained to each of the odorants in turn, finishing up with the labelling reagent, the presentation of which constituted the next "period" of labelling. These EOGs indicated the responsiveness of the epithelium after the first period of labelling.

The sequence of events was then repeated as often as necessary.
The applied pulse lengths of the vapour-phase labelling reagents were often varied between experiments and between reactive odorants. Consequently, the recovery times allowed after pulsing were altered accordingly (i.e. the longer the period of pulsing, the longer the recovery time employed). The results presented graphically in this chapter show ordinates which represent the EOG peak amplitudes obtained as a percentage of the original mean "100% value" and abscissae which indicate the total time (i.e. cumulative time) that the olfactory epithelium was exposed to the labelling odorant. Thus, the results are presented as time courses of labelling; they cover the periods within which the limit of EOG inhibition was achieved for each odorant under examination. It should be noted, however, that the abscissae do not reflect preparation longevity (see Section 4.3(i)); this tended to vary between animals after maximum labelling had been obtained.

Throughout the series of experiments, the olfactometer settings controlling the odorant flow rates were kept constant; vapour-phase odorant concentrations were routinely measured as previously described (see Section 2.4(ii)c). Tissue fatigue (adaptation) in the frog was avoided by permitting a 3 minute recovery period between stimulus presentations of 10 seconds duration; EOGs were recorded every minute from the in vitro rat preparation in response to 1 second odour pulses.

The camphoraceous odorant, 1,8-cineole was included in all the vapour-phase labelling experiments in order to provide a reference point for the many different investigations; its structure (Figure 3.5) and odour quality are unrelated to any of the other odorants utilised (see Squirrell, 1978).

1 1,3,3-trimethyl-2-cxabicyclo[2.2.2]octane.
Figure 3.5 Structure of the camphoraceous odorant 1,8-cineole
3.2(ii) **Experimental strategy**

The original aim of the present studies was the identification of putative olfactory receptor sites directed against pyrazine odorants: "if well-defined odours bind specifically to a single class of receptor protein with a high affinity, it should be possible to inactivate this class of protein without affecting the activity of the other classes of olfactory proteins" (Dodd and Persaud, 1981). Additionally, it was hoped to detect any interactions between the receptors that may contribute to the quality coding mechanism.

At the outset, pyrazine odorants (see Section 1.4(ii)) appeared to be the most suitable compounds for the attempted identification of independent receptor types\(^1\) (see Section 3.2(iii)). However, such identification proved unsuccessful; the EOGs recorded to a variety of odorants were differentially inhibited by pyrazine labelling reagents (see Figures 3.15 - 3.16). Consequently, the area of study was broadened to include examinations of the effects of other classes of vapour-phase labels on the EOG responses obtained to a variety of test odorants (Table 3.1); in the absence of specific labelling, all of these studies sought to demonstrate patterns of differential labelling. Such labelling profiles preclude receptor identification *per se*: "large-scale mapping experiments with many odorants would be required to identify the selectivity of the proteins" (Dodd and Persaud, 1981). However, these investigations may provide useful pointers for future examinations of labelling specificity.

Once this has been established, such studies should facilitate the

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\(^1\) Since 2-acetyl-3-methylpyrazine consistently failed to elicit an EOG response in the frog (see Section 3.3(ii)a), preliminary examinations of labelling efficiency employed readily available phenacyl compounds (see Section 3.3(1)a).
Table 3.1  Vapour-phase labelling experiments performed on the *in vivo* frog preparation and *in vitro* rat preparation

<table>
<thead>
<tr>
<th>Test odorants</th>
<th>phenacetyl bromide</th>
<th>phenacetyl chloride</th>
<th>3-pyridine-methanol bromo-acetate</th>
<th>2-pyridine-methanol bromo-acetate</th>
<th>3-bromo-acetyl pyridine</th>
<th>2-bromo-acetyl pyrazine</th>
<th>2-bromo-acetyl-3-methyl pyrazine</th>
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<tbody>
<tr>
<td>acetophenone</td>
<td>R</td>
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<td>R</td>
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<td>2-acetyl-3-methylpyrazine</td>
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<td>2-acetylpyrazine</td>
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<td>3-acetylpyridine</td>
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<tr>
<td>2-bromoacetyl-3-methylpyrazine</td>
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<td>2-bromoacetylpyrazine</td>
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<td>3-bromoacetylpyridine</td>
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<tr>
<td>1,8-cineole</td>
<td>F; R</td>
<td>F; R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
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<td>citronellol</td>
<td>R</td>
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<td>3,5-diteriarybutylacetophenone</td>
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<tr>
<td>ethyl 2-pyrazine carboxylate</td>
<td>R</td>
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<tr>
<td>isovaleryl acetate</td>
<td>F; R</td>
<td>F; R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
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<td>2-isopropyl-3-methoxypyrazine</td>
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<td>n-octanethiol</td>
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<td>phenacetyl bromide</td>
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<td>F; R</td>
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<tr>
<td>phenacyl chloride</td>
<td>F; R</td>
<td>F; R</td>
<td>R</td>
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<td>propiophenone</td>
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<td>2-pyridinemethanol bromoacetate</td>
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<td>3-pyridinemethanol bromoacetate</td>
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</tbody>
</table>

This table indicates the test odorants employed to elucidate the receptor specificity of each of the vapour-phase labelling odorants in the in vivo frog preparation (F) and/or the in vitro rat preparation (R).
isolation of receptor proteins; by labelling the appropriate binding sites with a radioactive version of the reactive odorant, the proteins will be "tagged" in situ, thereby aiding identification throughout subsequent isolation procedures (Dodd and Persaud, 1981).

3.2(iii) Choice of odorants

Attempts to characterise and identify receptor sites through the combined use of biochemical and electrophysiological methods on the vertebrate olfactory mucosa require a judicious choice of experimental odorants. Ideally, such compounds should possess the following features:

(a) low olfactory thresholds, indicating a probable high affinity for the binding sites

(b) a range of readily available congeners and derivatives for which it would be feasible to synthesise:

(c) (i) a radioactive derivative for ligand binding studies, and
(ii) a reactive derivative for chemical modification studies.

In general, pyrazines were regarded as the compounds of choice for the present investigations; members of this odorant class (some of which are important naturally-occurring compounds possessing a pervasive "green" odour; see Section 1.4(ii)) have some of the lowest olfactory thresholds known (e.g. 2-isobutyl-3-methoxypyrazine; 0.002 p.p.b.). Their employment in chemical modification studies was further supported by the possession of rigid molecular structures. Moreover, the availability of [3H]2-isobutyl-3-methoxypyrazine fulfilled the need for a radioactive odorant that could be used in ligand binding studies (see Chapter 6).

All of the odorants employed in the present chemical modification studies were required to elicit good quality EOG signals. However, it should be noted that the best responses were not always
obtained to the most interesting odorants, and vice versa: for example, larger EOG signals were evoked by acetophenone, which is not a particularly effective odorant, than by many of the heterocycles investigated.

Compounds suitable for derivitisation to vapour-phase labelling reagents\(^1\) were determined by their possession of a moiety which could be readily converted to a bromoacetyl or bromoacetate\(^2\) group (see Section 3.1(ii)a). This constraint on the choice of potential labels precluded the use of several aroma compounds (e.g. 4-butyl-5-propylthiazole; 2-isobutyl-3-methoxypyrazine) which, from other standpoints, appeared particularly interesting as odorants. Consequently, the odorants employed as labelling reagents in the present studies might not be regarded as the natural choice for other types of investigations (e.g. ligand binding studies) on the vertebrate olfactory system.

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1 It is difficult to accurately ascribe odour qualities to chemically reactive derivatives, since they tend to elicit a trigeminal reaction as well as an olfactory response; such compounds are often perceived as pungent, stinging sensations.

2 The previously performed vapour-phase labelling study of the fruity receptor site in the frog olfactory epithelium employed alkyl haloacetates (e.g. ethyl bromoacetate) as chemically reactive derivatives (Menevse et al, 1977b; Squirrell, 1978).
3.3 Results

The accompanying results show the mean and standard error of the data accumulated from single experiments on separate animals; the number of animals (n) used in each study is indicated.

3.3(i) Vapour-phase labelling studies on the in vivo frog preparation

Prior to the examinations described herein most chemical modification studies had been performed on in vivo frog preparations (e.g. see Getchell, 1971; Getchell and Gesteland, 1972; Menevse, 1977; Menevse et al., 1977b, 1978; Squirrell, 1978); little detailed experimentation had been carried out on the rat (but see Gesteland and Sigwart, 1977). Consequently, the present investigations focussed initially upon this in vivo frog preparation. These studies, which may be compared with earlier examinations of similar design, aimed to provide a reference point for the proposed work on the less-well-characterised in vitro rat preparation.

3.3(i)a Comparative effects of phenacyl bromide and phenacyl chloride on EOG responses obtained from the in vivo frog preparation

The effects exerted by these reactive derivatives of acetophenone on the EOGs obtained to several odorants were examined.

The experimental conditions were kept constant throughout the series of experiments, and the settings on the olfactometer were adjusted such that the flow rates of air through the U-tubes containing the reactive odorants were identical.

The results (Figures 3.6 and 3.7) show that a selective reduction in the EOGs obtained to propiophenone could be brought about in the frog by utilising phenacyl bromide as a vapour-phase labelling reagent; the EOG responses to the structurally unrelated odorants, isoamyl acetate and 1,8-cineole, remained largely unaffected.\(^1\)

\(^1\) A slight increase in these responses was found after limited exposure of the epithelium to phenacyl chloride. Increased responses following treatment with ethyl chloroacetate have been previously reported (Squirrell, 1978).
These figures, which represent time courses of labelling, are presented in the form shown below. The duration of each period of labelling may be estimated by reference to the intervals between successive experimental points (here indicated by the letters a - f).

The recovery time permitted after each aliquot of labelling, prior to the recording of further ECG responses, depended upon the pulse length of the reactive odorant, as follows:

<table>
<thead>
<tr>
<th>Pulse length of labelling reagent (sec.)</th>
<th>Recovery time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td>60</td>
<td>15</td>
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</table>

Each figure states the vapour-phase concentration of the labelling reagent employed.

ECG responses to non-reactive odorants were obtained (a) at 3 minute intervals from the in vivo frog preparation in response to 1C second stimulus presentations, and (b) at 1 minute intervals from the in vitro rat preparation in response to 1 second odor pulses.
Figure 3.6  Vapour-phase labelling of frog olfactory epithelium with phenacyl bromide

EOG peak amplitude (% of original mean value)

- isomyl acetate
- 1,8-cineole
- propiophenone
- phenacyl bromide (0.35µM)  n = 8

Cumulative time of labelling (min.)

Figure 3.7  Vapour-phase labelling of frog olfactory epithelium with phenacyl chloride

EOG peak amplitude (% of original mean value)

- 1,8-cineole
- isomyl acetate
- propiophenone
- phenacyl chloride (0.41µM)  n = 7

Cumulative time of labelling (min.)
When phenacyl chloride was employed as the labelling odorant, this preferential reduction was considerably diminished (Table 3.2).

The comparatively high degree of selective labelling obtained with phenacyl bromide\(^2\) suggested that this compound, together with other brominated derivatives should be employed in the proposed vapour-phase labelling experiments on the in vitro rat preparation.

3.3(i)b Concentration dependence of vapour-phase labelling with phenacyl bromide

The in vivo frog preparation was pulsed with different concentrations of phenacyl bromide (Figure 3.8); the EOG peak amplitudes remaining after three minutes\(^1\) (cumulative time) exposure of the epithelium to various concentrations of the labelling reagent are shown in Table 3.3.

The results indicate that, in the frog, vapour-phase labelling with phenacyl bromide is concentration-dependent. Similar findings for the labelling produced by ethyl bromoacetate have been previously reported (Squirrell, 1978).

---

\(^2\) In addition, phenacyl bromide proved to be stable for several weeks when dissolved in diethyl phthalate and stored in the dark at room temperature.
Table 3.2  Comparative effects of phenacyl bromide and phenacyl chloride on EOG responses obtained from the in vivo frog preparation

<table>
<thead>
<tr>
<th>Odorant</th>
<th>Vapour-phase labelling reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EOG peak amplitude (% of original mean value) after 5 minutes (cumulative time) labelling (mean ± S.E.M.)</td>
</tr>
<tr>
<td></td>
<td>Phenacyl bromide (0.35μM; n = 8)</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>94 ± 3</td>
</tr>
<tr>
<td>isoamyl acetate</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>propiophenone</td>
<td>54 ± 5</td>
</tr>
<tr>
<td>phenacyl halide</td>
<td>34 ± 6</td>
</tr>
</tbody>
</table>

Tabular representation of some of the data presented in Figure 3.6 and Figure 3.7 showing the mean ± S.E.M. values of the EOG peak amplitudes (expressed as a % of the original mean baseline response) obtained after exposing the frog olfactory epithelium to phenacyl bromide or phenacyl chloride for 5 minutes (cumulative time).
Figure 3.8  Vapour-phase labelling of frog olfactory epithelium with different concentrations of phenacyl bromide

(a) EOG peak amplitude (% of original mean value)
- isoamyl acetate
- 1,8-cineole
- propiophenone
- phenacyl bromide (0.35μM) n = 8

(b) EOG peak amplitude (% of original mean value)
- 1,8-cineole
- isoamyl acetate
- propiophenone
- phenacyl bromide (0.18μM) n = 4

(c) EOG peak amplitude (% of original mean value)
- isoamyl acetate
- 1,8-cineole
- propiophenone
- phenacyl bromide (0.10μM) n = 4

Cumulative time of labelling (min)

(Presented previously as Figure 3.6)
Table 3.3  Effects of different concentrations of phenacyl bromide on EOG responses obtained from the in vivo frog preparation

<table>
<thead>
<tr>
<th>Vapour-phase concentration of phenacyl bromide (µM)</th>
<th>Odorant</th>
<th>1,8-Cineole</th>
<th>Isoamyl acetate</th>
<th>Propio-phenone</th>
<th>Phenacyl bromide</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.35 (n = 8)</td>
<td></td>
<td>95 ± 3</td>
<td>96 ± 2</td>
<td>59 ± 4</td>
<td>37 ± 4</td>
</tr>
<tr>
<td>0.18 (n = 4)</td>
<td></td>
<td>97 ± 2</td>
<td>99 ± 2</td>
<td>79 ± 3</td>
<td>62 ± 5</td>
</tr>
<tr>
<td>0.10 (n = 4)</td>
<td></td>
<td>99 ± 2</td>
<td>98 ± 2</td>
<td>94 ± 3</td>
<td>81 ± 3</td>
</tr>
</tbody>
</table>

Tabular representation of some of the data presented in Figure 3.8 showing the mean ± S.E.M. values of the EOG peak amplitudes (expressed as a % of the original mean baseline response) obtained after exposing the frog olfactory epithelium to each of three different concentrations of phenacyl bromide for 3 minutes (cumulative time).
3.3(ii) Vapour phase labelling studies on the in vitro rat preparation

3.3(ii)a Effects of phenacyl bromide, phenacyl chloride, 3-pyridinemethanol bromoacetate, 2-pyridinemethanol bromoacetate, 3-bromoacetyl pyridine, 2-bromoacetylpyrazine and 2-bromoacetyl-3-methylpyrazine on EOG responses obtained from the in vitro rat preparation

Initial studies (Figures 3.9 and 3.10) reproduced the findings of the in vivo frog examinations (see Section 3.3(i)); a comparison of the extents to which the EOG responses to propiophenone were selectively inhibited (which are similar to those observed with the frog; see Table 3.2) shows phenacyl bromide to be the more effective vapour-phase labelling reagent (Table 3.4). The results (Table 3.5) also indicate that, in the rat, such labelling is concentration-dependent (also see Section 3.3(i)b).

These findings suggested that other brominated odorants might also preferentially (or specifically) and irreversibly reduce the EOG responses obtained to their non-reactive analogues. To this end, the specificity of labelling produced by six potential labels (including phenacyl bromide) was examined by monitoring their effects on the EOGs obtained to a variety of odorants (see Table 3.1). Since the labelling behaviour of a reactive odorant reflects its specificity of binding, it was hoped that these studies would furnish various patterns of EOG inhibition, thus providing independent controls for the separate investigations.

In general, the results (Figures 3.11 - 3.16) show that each of the labelling reagents differentially inhibited the EOGs obtained to several odorants, with the greatest inhibitory effects being exerted upon the responses obtained to themselves\(^1\); the EOGs

\(^1\) The level of inhibition routinely observed (about 65%) was comparable with that found for the EOGs obtained to small fatty acids after treatment of the frog or rat olfactory mucosa with concanavalin A (see Chapter 5).
Figure 3.9 Vapour-phase labelling of rat olfactory epithelium with two concentrations of phenacyl bromide

(a)

ECG peak amplitude (% of original mean value)

- isoamyl acetate
- 1,8-cineole
- propiophenone
- phenacyl bromide (0.35μM) n = 6

Cumulative time of labelling (min)

(b)

ECG peak amplitude (% of original mean value)

- isoamyl acetate
- 1,8-cineole
- propiophenone
- phenacyl bromide (0.10μM) n = 5

Cumulative time of labelling (min).
Figure 3.10  Vapour-phase labelling of rat olfactory epithelium with phenacyl chloride

![Graph showing vapour-phase labelling of rat olfactory epithelium with phenacyl chloride.](image)

- EOG peak amplitude (% of original mean value)
- Cumulative time of labelling (min)

- 1,8-cineole
- isoamyl acetate
- propiophenone
- phenacyl chloride (0.41μM)  n = 4
Table 3.4  Comparative effects of phenacyl bromide and phenacyl chloride on EOG responses obtained from the in vitro rat preparation

<table>
<thead>
<tr>
<th>Odorant</th>
<th>Vapour-phase labelling reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phenacyl bromide (0.35µM; n=6)</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>96 ± 4</td>
</tr>
<tr>
<td>isoamyl acetate</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>propiophenone</td>
<td>51 ± 6</td>
</tr>
<tr>
<td>phenacyl halide</td>
<td>29 ± 8</td>
</tr>
</tbody>
</table>

Tabular representation of some of the data presented in Figure 3.9 and Figure 3.10 showing the mean ± S.E.M. values of the EOG peak amplitudes (expressed as a % of the original mean baseline response), obtained after exposing the rat olfactory epithelium to phenacyl bromide or phenacyl chloride for 5 minutes (cumulative time).
Table 3.5 Effects of different concentrations of phenacyl bromide on EOG responses obtained from the in vitro rat preparation

<table>
<thead>
<tr>
<th>Vapour-phase concentration of phenacyl bromide (µM)</th>
<th>Odorant</th>
<th>EOG peak amplitude (% of original mean value) after 3 minutes (cumulative time) labelling (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1,8-Cineole</td>
<td>Isoamyl acetate</td>
</tr>
<tr>
<td>0.35 (n = 6)</td>
<td>98 ± 3</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>0.18 (n = 5)</td>
<td>99 ± 2</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>0.10 (n = 5)</td>
<td>99 ± 2</td>
<td>100 ± 1</td>
</tr>
</tbody>
</table>

Tabular representation of data (some of which is taken from Figure 3.9) showing the mean ± S.E.M. values of the EOG peak amplitudes (expressed as a % of the original mean baseline response) obtained after exposing the rat olfactory epithelium to each of three different concentrations of phenacyl bromide for 3 minutes (cumulative time).
Figure 3.11  Vapour-phase labelling of rat olfactory epithelium with phenacyl bromide

(a) 1,8-cineole  
○ 3,5-diteriarybutylacetophenone  
● acetophenone  
Δ propiophenone  
* phenacyl bromide (0.35μM) n = 7

Cumulative time of labelling (min)

(b) 1,8-cineole  
○ citronellol  
● isovaleric acid  
Δ propiophenone  
* phenacyl bromide (0.35μM) n = 6

Cumulative time of labelling (min)

(c) 1,8-cineole  
○ trimethylpyrazine  
● 3-pyridinemethanol propionate  
○ ethyl 2-pyrazinecarboxylate  
* phenacyl bromide (0.35μM) n = 7

Cumulative time of labelling (min)
Figure 3.12 Vapour-phase labelling of rat olfactory epithelium with 3-pyridinemethanol bromoacetate

(a)

EOG peak amplitude (% of original mean value)

- isovaleryl acetate  n = 8
- 1,8-cineole
- acetophenone
- propiophenone
- 3-pyridinemethanol propionate

Cumulative time of labelling (min)

(b)

EOG peak amplitude (% of original mean value)

- 1,8-cineole  n = 8
- isovaleric acid
- ethyl 2-pyrazinecarboxylate
- 3-pyridinemethanol propionate
- 3-pyridinemethanol bromoacetate (0.38 μM)

Cumulative time of labelling (min)
Figure 3.13 Vapour-phase labelling of rat olfactory epithelium with 2-pyridinemethanol bromoacetate

![Graph showing EOG peak amplitude (% of original mean value) vs cumulative time of labelling (min) for different substances including isoamyl acetate, 1,8-cineole, ethyl 2-pyrazine-carboxylate, 3-pyridinemethanol propionate, 2-pyridinemethanol propionate, and 2-pyridinemethanol bromoacetate. Each substance is represented by a line on the graph, with data points indicating the EOG peak amplitude at different cumulative times. The graph shows that 2-pyridinemethanol bromoacetate has the highest EOG peak amplitude among the substances tested.](image-url)
Figure 3.14  Vapour-phase labelling of rat olfactory epithelium with 3-bromoacetylpyridine

(a)  
EOG peak amplitude (% of original mean value) 

- isoamyl acetate  
- 3-pyridinemethanol propionate  
- 3-acetylpyridine  
- propiophenone  

n = 7  
Cumulative time of labelling (min)

(b)  
EOG peak amplitude (% of original mean value) 

- 1,8-cineole  
- isovaleric acid  
- octanethiol  
- 3-acetylpyridine  
- 3-bromoacetylpyridine (0.30μM)  

n = 7  
Cumulative time of labelling (min)
Figure 3.15: Vapour-phase labelling of rat olfactory epithelium with 2-bromoacetylpyrazine

(a) EOG peak amplitude (% of original mean value)

- isoamyl acetate
- 1,8-cineole
- octanethiol
- 2-acetyl-3-methylpyrazine
- 2-bromoacetylpyrazine (0.21 µM) n = 8

Cumulative time of labelling (min)

(b) EOG peak amplitude (% of original mean value)

- isoamyl acetate
- 1,8-cineole
- propiophenone
- 2-acetylpyrazine
- 2-bromoacetylpyrazine (0.21 µM) n = 8

Cumulative time of labelling (min)
Figure 3.16  Vapour-phase labelling of rat olfactory epithelium with 2-bromoacetyl-3-methylpyrazine

(a)

ECG peak amplitude (% of original mean value)

- 1,8-cineole
- 2-isopropyl-3-methoxypyrazine
- 2-acetylpyrazine
- 2-acetyl-3-methylpyrazine
- 2-bromoacetyl-3-methylpyrazine (0.28µM)

Cumulative time of labelling (min)

(b)

EOG peak amplitude (% of original mean value)

- isoamyl acetate
- 1,8-cineole
- acetophenone
- propiophenone
- 2-acetyl-3-methylpyrazine

Cumulative time of labelling (min)
to odorants possessing molecular structures and/or odour qualities that differed greatly from the labelling reagents (e.g. isoamyl acetate; 1,8-cineole; isovaleric acid; n-octanethiol etc.) remained largely unaffected. A similar degree of EOG inhibition (see Table 3.6) was experienced by each of the six non-reactive analogues (e.g. propiophenone; 3-pyridinemethanol propionate; 2-acetylpyrazine etc.).

Several other observations are also worthy of note. Studies employing phenacyl bromide showed that the EOGs obtained to 3,5-ditertiarybutylacetophenone (which is bulkier than its parent compound) were less inhibited by the labelling reagent than were those obtained to acetophenone and to propiophenone (Table 3.7). The similar degree of labelling experienced by these two odorants demonstrates that such EOG inhibition is not significantly influenced by slight variations in the size of the acyl moiety (Table 3.7); this observation is important since it serves to validate the studies which employ α-brominated derivatives of acetyl heterocycles (rather than compounds containing a bromoacetate group; see Section 3.1(ii)a) as vapour-phase labelling reagents (e.g. 3-bromoacetylpyridine). Similar findings have been previously reported: the EOGs obtained from the frog to ethyl acetate and to ethyl propionate were inhibited "by very similar degrees" (51.1% and 49.7% respectively) after labelling with ethyl bromoacetate (Squirrell, 1978).

Heterocyclic odorants tend to possess highly developed structure-activity relationships (e.g. see Pittet and Hruza, 1974; Teranishi et al., 1974; also see Section 1.4(ii)); small variations in their molecular structures may impart different sensory properties and ultimately affect the specificity of binding to olfactory
Table 3.6  Comparative effects of vapour-phase labelling reagents on EOG responses obtained to themselves and to their non-reactive analogues from the in vitro rat preparation

<table>
<thead>
<tr>
<th>Vapour-phase labelling reagent</th>
<th>Vapour-phase labelling reagent</th>
<th>Non-reactive analogue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenacyl bromide (0.35µM; n=6)</td>
<td>Phenacyl bromide 36 ± 8</td>
<td>Propiophenone 54 ± 5</td>
</tr>
<tr>
<td>3-pyridinemethanol bromoacetate (0.38µM; n=8)</td>
<td>3-pyridinemethanol bromoacetate 34 ± 8</td>
<td>3-pyridinemethanol propionate 48 ± 4</td>
</tr>
<tr>
<td>2-pyridinemethanol bromoacetate (0.36µM; n=6)</td>
<td>2-pyridinemethanol bromoacetate 28 ± 8</td>
<td>2-pyridinemethanol propionate 51 ± 8</td>
</tr>
<tr>
<td>3-bromoacetylpyridine (0.30µM; n=7)</td>
<td>3-bromoacetylpyridine 33 ± 7</td>
<td>3-acetylpyridine 64 ± 6</td>
</tr>
<tr>
<td>2-bromoacetylpyrazine (0.21µM; n=8)</td>
<td>2-bromoacetylpyrazine 36 ± 8</td>
<td>2-acetylpyrazine 56 ± 8</td>
</tr>
<tr>
<td>2-bromoacetyl-3-methylpyrazine (0.28µM; n=9)</td>
<td>2-bromoacetyl-3-methylpyrazine 31 ± 9</td>
<td>2-acetyl-3-methylpyrazine 57 ± 7</td>
</tr>
</tbody>
</table>

Tabular representation of some of the data presented in Figures 3.11 - 3.16, showing the mean ± S.E.M. values of the EOG peak amplitudes (expressed as a % of the original mean baseline response) obtained to phenacyl bromide, 3-pyridinemethanol bromoacetate, 2-pyridinemethanol bromoacetate, 3-bromoacetylpyridine, 2-bromoacetylpyrazine and 2-bromoacetyl-3-methylpyrazine and to their non-reactive analogues after exposing the rat olfactory epithelium to each of the six vapour-phase labelling reagents for 5 minutes (cumulative time).
Table 3.7
Differential effect of phenacyl bromide on EOG responses obtained from the in vitro rat preparation

<table>
<thead>
<tr>
<th>Odorant</th>
<th>Inhibition of EOG peak amplitude (% of original mean value) after 5 minutes (cumulative time) labelling (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,8-cineole (n = 7)</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>trimethylpyrazine (n = 7)</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>citronellol (n = 6)</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>isovaleric acid (n = 6)</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>3-pyridinemethanol propionate</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>3,5-diteriarybutylacetophenone</td>
<td>17 ± 5</td>
</tr>
<tr>
<td>ethyl 2-pyrazine-carboxylate (n = 7)</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>acetophenone (n = 7)</td>
<td>42 ± 6</td>
</tr>
<tr>
<td>propiophenone (n = 7)</td>
<td>49 ± 7</td>
</tr>
<tr>
<td>phenacyl bromide (0.35 µM) (n = 7)</td>
<td>64 ± 8</td>
</tr>
</tbody>
</table>

Tabular representation of some of the data presented in Figure 3.11 showing the mean ± S.E.M. values of the inhibition of EOG peak amplitudes (expressed as a % of the original mean baseline response) obtained after exposing the rat olfactory epithelium to phenacyl bromide for 5 minutes (cumulative time).
receptor sites. The present findings are compatible with such ideas; the ECG responses to ethyl 2-pyrazinecarboxylate were less inhibited by 3-pyridinemethanol bromoacetate than were those obtained to 3-pyridinemethanol propionate (Table 3.8; Figure 3.12); the addition of a methyl group to 2-acetylpyrazine considerably reduced the extent to which the resulting odorant was labelled by 2-bromoacetylpyrazine (Table 3.8; Figure 3.15); the ECGs obtained to ethyl 2-pyrazinecarboxylate and to 3-pyridinemethanol propionate were less inhibited by 2-pyridinemethanol bromoacetate than were those recorded to 2-pyridinemethanol propionate (Table 3.9; Figure 3.13); the responses obtained to 2-isopropyl-3-methoxypyrazine and to 2-acetylpyrazine were less inhibited by 2-bromoacetyl-3-methyl pyrazine than were the ECGs to 2-acetyl-3-methylpyrazine (Table 3.9; Figure 3.16), and finally, the EOG responses to ethyl 2-pyrazine-carboxylate and to 3-pyridinemethanol propionate were less inhibited by phenacyl bromide than were those obtained to acetophenone and to propiophenone (Table 3.7; Figure 3.11).

It is worthy of note that all attempts (albeit made within a 5 month period) to elicit an EOG response in the frog to 2-acetyl-3-methylpyrazine consistently failed, even though small stable responses (about 1.5 mV) were obtained with the in vitro rat preparation (see Figure 3.17). Although not rigorously investigated this observation is interesting since no reports have appeared elsewhere of frogs (unlike mice; see Wysocki, 1984) being insensitive to any one particular odorant.
Table 3.8  Differential effects of 3-pyridinemethanol bromoacetate and of 2-bromoacetylpyrazine on EOG responses obtained from the in vitro rat preparation

(a)

<table>
<thead>
<tr>
<th>Odorant</th>
<th>Inhibition of EOG peak amplitude (% of original mean value) after 5 minutes (cumulative time) labelling (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ethyl 2-pyrazine-carboxylate (n = 8)</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>3-pyridinemethanol propionate (n = 8)</td>
<td>52 ± 4</td>
</tr>
<tr>
<td>3-pyridinemethanol bromoacetate (0.38µM; n = 8)</td>
<td>66 ± 8</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Odorant</th>
<th>Inhibition of EOG peak amplitude (% of original mean value) after 5 minutes (cumulative time) labelling (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-acetyl-3-methylpyrazine (n = 8)</td>
<td>24 ± 9</td>
</tr>
<tr>
<td>2-acetylpyrazine (n = 8)</td>
<td>44 ± 8</td>
</tr>
<tr>
<td>2-bromoacetylpyrazine (0.24µM; n = 8)</td>
<td>64 ± 8</td>
</tr>
</tbody>
</table>

Tabular representation of some of the data presented in (a) Figure 3.12 and (b) Figure 3.15 showing the mean ± S.E.M. values of the inhibition of EOG peak amplitudes (expressed as a % of the original mean baseline response) obtained to selected odorants after exposing the rat olfactory epithelium to (a) 3-pyridinemethanol bromoacetate and (b) 2-bromoacetylpyrazine for 5 minutes (cumulative time).
Table 3.9  Differential effects of 2-pyridinemethanol bromoacetate and of 2-bromoacetyl-3-methylpyrazine on EOG responses obtained from the in vitro rat preparation

(a)

<table>
<thead>
<tr>
<th>Odorant</th>
<th>Inhibition of EOG peak amplitude (% of original mean value) after 5 minutes (cumulative time) labelling (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ethyl 2-pyrazine-carboxylate (n = 6)</td>
<td>18 ± 5</td>
</tr>
<tr>
<td>3-pyridinemethanol propionate (n = 6)</td>
<td>28 ± 6</td>
</tr>
<tr>
<td>2-pyridinemethanol propionate (n = 6)</td>
<td>49 ± 8</td>
</tr>
<tr>
<td>2-pyridinemethanol bromoacetate (0.36μM) (n = 6)</td>
<td>72 ± 8</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Odorant</th>
<th>Inhibition of EOG peak amplitude (% of original mean value) after 5 minutes (cumulative time) labelling (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-isopropyl-3-methoxy-pyrazine (n = 9)</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>2-acetylpyrazine (n = 9)</td>
<td>21 ± 6</td>
</tr>
<tr>
<td>2-acetyl-3-methyl-pyrazine (n = 9)</td>
<td>43 ± 7</td>
</tr>
<tr>
<td>2-bromoacetyl-3-methylpyrazine (0.26μM) (n = 9)</td>
<td>69 ± 9</td>
</tr>
</tbody>
</table>

Tabular representation of some of the data presented in (a) Figure 3.13 and (b) Figure 3.16 showing the mean ± S.E.M. values of the inhibition of EOG peak amplitudes (expressed as a % of the original mean baseline response) obtained to selected odorants after exposing the rat olfactory epithelium to (a) 2-pyridinemethanol bromoacetate and (b) 2-bromoacetyl-3-methylpyrazine for 5 minutes (cumulative time).
Figure 3.17 Electrophysiological recordings from the in vitro rat preparation and in vivo frog preparation following their exposure to 2-acetyl-3-methylpyrazine

(a)

The upper recording (a) shows a typical ECG obtained from the in vitro rat preparation to 2-acetyl-3-methylpyrazine (0.27 μM), whilst the lower trace (b) shows that no such signal was obtained from the in vivo frog preparation following its exposure to this odourant. The horizontal bars indicate stimulus duration.
Protection experiments on the in vitro rat preparation

The demonstration of protection is an important criterion for establishing the occurrence of affinity labelling (Singer, 1970); the rate of specific inactivation of a particular binding site should be reduced in the presence of an appropriate ligand.

The present studies attempted to specifically protect rat olfactory receptor sites against vapour-phase labelling with 2-bromoacetyl-3-methylpyrazine. The methodology was developed from that used previously in similar studies on the in vivo frog preparation (see Squirrell, 1978). The olfactory epithelium was exposed to 2-acetyl-3-methylpyrazine (0.3 µmoles/litre of air) for a period of 10 seconds; this odorant acted as the protectant. Next, the odor applicator was switched such that the tissue was pulsed with 2-bromoacetyl-3-methylpyrazine (0.3 µmoles/litre of air) in the presence of protectant (3.4 µmoles/litre of air); the reactive odorant was dissolved in diethyl phthalate and housed with the protectant in a U-tube. After each "aliquot" of labelling, the odor applicator was switched back to the original position, and the olfactory tissue was exposed to 2-acetyl-3-methylpyrazine for a further period of 10 seconds. The overall effect of this procedure was to pulse the olfactory epithelium with various aliquots of the vapour-phase labelling odorant whilst all the time exposing it to 2-acetyl-3-methylpyrazine.

The results (Figures 3.18 and 3.19) show that the rate of specific inhibition of the EOGs to 2-acetyl-3-methylpyrazine was reduced when this odorant was applied to the rat olfactory epithelium before, during and after treatment with the vapour-phase label 2-bromoacetyl-3-methylpyrazine. This observation is consistent with the appearance of specific protection (also see Squirrell, 1978;
The protectant, 2-acetyl-3-methylpyrazine (3.38 μM), was applied to the rat olfactory epithelium before, during and after exposure of the tissue to 2-bromoacetyl-3-methylpyrazine (0.28 μM).
The rat olfactory epithelium was exposed to humidified air devoid of protectant before, during and after the application of 2-bromoacetyl-3-methylpyrazine (0.28 μM) (also see Figure 3.16)
Menevse et al., 1978). It should be noted, though, that an unequivocal demonstration of such behaviour requires the employment of protectant analogues. However, the difficulties encountered in performing protection experiments on the vertebrate olfactory system (e.g. adaptation; effect on the epithelium of prolonged exposure to odorants (see Section 4.3(iv)); possible odorant metabolism by nasal cytochrome P-450-dependent monoxygenases (see Section 1.3(ii)c)), which are greater than those found with many other types of receptors, hinders the execution of such studies. Consequently, the accompanying results should only be regarded as being compatible with, and not unambiguously indicative of, the occurrence of specific protection.

The intense stimulations of 2-acetyl-3-methylpyrazine used throughout these experiments precluded the measurement of EOG responses to 2-bromoacetyl-3-methylpyrazine. Following treatment, a rest period of 10 - 15 minutes (depending upon the pulse length of the reactive odorant) permitted recovery from the effects of adaptation.

Control experiments for these protection studies were performed by replacing the protectant (i.e. 2-acetyl-3-methylpyrazine)-carrying air stream with an equal volume of humidified air (i.e. the incoming air stream was passed through a U-tube containing only diethyl phthalate), and by pulsing the tissue with only 2-bromoacetyl-3-methylpyrazine. In this case, the EOG to the reactive odorant could be recorded. Again, a rest period (about 10 minutes) preceded the recording of any further EOG responses. The settings on the olfactometer (i.e. flow restrictors) were kept constant between these experiments and the protection investigations.
3.3(ii)c Recovery of EOG responses

After treating the rat olfactory epithelium with any of the vapour-phase labelling odorants employed in the present studies, there was no observable recovery of the EOG response within several hours. Thus, the labelling produced appears to be irreversible. Similar findings have been reported for the vapour-phase labelling of frog olfactory receptors with haloalkyl esters (see Squirrell, 1978).

3.3(ii)d Appearance of EOG responses

Some EOG signals from a typical vapour-phase labelling experiment on the in vitro rat preparation are shown in Figure 3.20. Squirrell (1978) has indicated that the waveform of the EOGs obtained from the frog to several haloalkyl esters was altered as a result of labelling; the amplitude of the plateau component of the EOG was more reduced than the amplitude of the initial peak, such changes being accompanied by the appearance of a small, negative, "off-response". However, the waveform of the EOG responses to various non-halogenated alkyl esters was not affected in this manner, 'perhaps because they had higher peak/plateau ratios to start with' (Squirrell, 1978).
Figure 3.20  Some EOG signals obtained from a typical vapour-phase labelling experiment on the in vitro rat preparation

(a)  

1,8-cineole

propiophenone isocamyl acetate

0.4 mV  20 sec.

(b)  

1,8-cineole

propiophenone isocamyl acetate

0.4 mV  20 sec.

These EOG signals were recorded (a) before and (b) after vapour-phase labelling (40 sec. cumulative time) of the rat olfactory epithelium with 2-bromoacetylpyrazine (0.21μM) (see Figure 3.15). The stimulus pulse was 1 sec., sufficient to elicit the EOG peak.
3.4 Discussion

The results obtained from the present studies appear generally consistent with the demonstration of affinity labelling; each of the labelling reagents preferentially inhibited the EOG responses to itself and to its non-reactive analogue (see Figures 3.11 - 3.16) whilst findings compatible with the appearance of specific protection were obtained using 2-acetyl-3-methylpyrazine (see Section 3.3(ii)b). However, the profiles do reveal possible inconsistencies with respect to such labelling.

Firstly, it should be noted that the kinetics of the EOG reductions varied between odorants, and in particular between the labelling reagents and their non-reactive analogues. Previous vapour-phase modification studies have given rise to similar effects (e.g. see Squirrell, 1978; Persaud, 1980). Since affinity odorants and analogues may be expected to display superimposable labelling kinetics, such disparities may indicate deviation from true affinity labelling. Conversely, however, the absence of coincidence may simply reflect concentration differences between the halogenated and non-reactive odorants, or their different degrees of binding to the mucus which overlies the olfactory mucosa (see Section 5.4).

Secondly, different extents of EOG inhibition were experienced by the labelling reagents (about 65% of the original value) and their non-reactive analogues (about 45% of the original value). This lack of superimposability, which has also been detected in previous vapour-phase labelling studies, suggests that the labelling odorants somehow affect (i.e. recruit) receptor sites that are unresponsive to the

---

1 In common with previous studies of similar design (e.g. see Squirrell, 1978; Persaud, 1980), this parameter (as reflected by the plateau regions of Figures 3.11 - 3.16) was routinely used as the indicator of labelling behaviour.
non-reactive analogues. However, such apparent discrepancies do not necessarily refute the thesis that true affinity labelling is being monitored; again, this behaviour may simply reflect differences in concentration between the halogenated and non-reactive odorants.

These labelling experiments conceal many technical difficulties which hinder their successful performance; mechanical and electrical problems, together with complications arising from the animal preparations themselves (e.g. variations in age, health, nutritional state, etc; ease of dissection; adaptation effects etc.) all contribute to the many problems frequently encountered in this type of study. Such difficulties inhibited any examination of the conditions under which optimum labelling may be observed; odorant concentrations were chosen solely on their ability to elicit good quality EOG responses (Menevse et al, 1978). Thus, no attempts were made to examine the concentration dependence of the EOG signals. It is possible therefore that the disparity in the extents (and kinetics) of labelling experienced by the various labelling reagents and by the non-reactive analogues may reflect their relative abilities to elicit good quality EOG responses at dissimilar odour concentrations.

Confirmation, or otherwise, of this hypothesis must await the

---

1 E.g. the incidence of 2-acetyl-3-methylpyrazine consistently failing to elicit an EOG response in the in vivo frog preparation (see Section 3.3(ii)a).

2 It should be noted that attempts are made to overcome these obstacles; as far as practicable, the experimental methodology controls for variations between the animal preparations and for any artifactual labelling behaviour (e.g. isoamyl acetate and 1,8-cineole are employed in all the experiments as "control" odours; the position of the recording microelectrode is standardised; operating conditions are kept constant between experiments, etc; also see Chapter 4).

3 Each odorant was used at the same (vapour-phase) concentration in every labelling experiment.
performance of further studies, including large scale mapping experiments with many odorants. The monitoring of single unit responses may prove advantageous to these examinations, although it should be noted that such recordings present greater technical challenges than those encountered with ensemble techniques.

The possible discrepancies that exist with respect to genuine affinity labelling \(^1\) do, however, complicate interpretation of the present studies. Consequently, these investigations have been termed "vapour-phase labelling studies" as opposed to "affinity labelling studies". It must be stressed, though, that the findings of differential ECG inhibition remain equally valid.

Preliminary studies on the \textit{in vivo} frog preparation and \textit{in vitro} rat preparation showed that phenacyl bromide was a more effective labelling reagent than phenacyl chloride. This finding is consistent both with previous observations on the relative abilities of ethyl chloroacetate, ethyl bromoacetate and ethyl iodoacetate to selectively reduce ECG responses obtained from the frog (Squirrell, 1978), and with the reported relative reactivity of haloacetates towards nucleophiles, which follows the order:

\[
\text{iodo-} > \text{bromo-} > \text{chloro-} > \text{fluoro-}
\]

(in the ratios 3 : 1 : 0.2 : 0.0001) (Hendrickson \textit{et al}, 1970; but also see Korman and Clarke, 1956). The employment of haloketones as affinity labelling reagents (Hartman, 1977), and the particular advantages applicable to the use of bromoacetyl reagents (Wilchek and Givol, 1977) have been discussed elsewhere.

The validity of the present vapour-phase labelling studies

\(^1\) It has been previously suggested that "it would be wise to refine the terminology regarding 'affinity labels'" for labelling studies employing halogenated esters (Karnovsky, 1961).
was indicated by the apparent demonstration of specific protection; the EOGs obtained to 2-acetyl-3-methylpyrazine were found to be protected when a high concentration of this odorant was present before, during and after exposure of the rat olfactory epithelium to 2-bromoacetyl-3-methylpyrazine. The significance for the odour quality coding mechanism in the olfactory primary neurones of the differential protection afforded by n-pentyl acetate to various odorants against labelling by the thiol-specific reagent, mersalyl, has been previously discussed by Menevse et al (1978).

The labelling effects of phenacyl bromide, 3-pyridinemethanol bromoacetate, 2-pyridinemethanol bromoacetate, 3-bromoacetylpyridine 2-bromoacetylpyrazine and 2-bromoacetyl-3-methylpyrazine on the ECG responses obtained to a variety of odorants were examined with the in vitro rat preparation. The results show that, in general, the EOGs to compounds resembling the labelling reagents (with respect to molecular structure and/or odour quality) were selectively reduced, whereas those evoked by odorants bearing little similarity to the reactive derivatives remained comparatively unaffected; relatively slight differences in odorant structure/quality were found to affect markedly the extents of observed EOG inhibition (e.g. see Figure 3.12: ethyl 2-pyrazinecarboxylate was inhibited to a much lesser degree by 3-pyridinemethanol bromoacetate than was 3-pyridinemethanol propionate).

These findings appear to support the notion that olfactory discrimination is attributable to the interaction of stimulus molecules with highly specific receptor sites contained within the vertebrate olfactory system (Amoore, 1970b; also see Section 1.3(ii)c). However, the failure to ever bring about ECG abolition tends to argue against this hypothesis and suggests that the labelling patterns
reflect the co-existence in the olfactory system of sites capable of binding one or more odorants with different affinities (i.e. the olfactory receptor sites possess a certain degree of overlapping specificity).

The interaction of odorants with more than one type of receptor site has been previously discussed (Beets, 1971; Polak, 1973); evidence on the response specificity of individual olfactory neurones suggests that these cells are generalist cells which respond in a differential manner to a wide range of odorants\(^1\) (Gesteland, 1976). This has been cited as a possible explanation for why odour primaries have so far been difficult to define: the primaries hitherto identified (e.g. see Amoore, 1971, 1974) may in fact be odorants that interact with only a few types of receptor sites, with one type predominating (Getchell and Getchell, 1971+).

It should be noted that the labelling obtained with the in vivo frog preparation (Figures 3.6 - 3.8) occurred at a slower rate than that previously reported using ethyl bromoacetate (Figure 3.3). Although this variation in rate may simply reflect differences between the halcalkyl ester and the phenacyl compounds presently employed, it should also be stressed that the two investigations utilised separate olfactometers and odour applicators; variations in their performance may account for the observed rate differences\(^2\).

---

1 It has been suggested that the odour reception of n-alcohols involves rather non-specific, probably hydrophobic, interaction (Senf et al, 1980; also see Cherry et al, 1970).

2 Separate olfactometers and odour applicators were also used in the present studies on the frog preparation and on the rat preparation. Again, variations in their performance may help to explain the differences observed between the rates of labelling obtained with these two preparations.
Most of the labelling profiles show a gradual, non-specific reduction with time in the EOG responses obtained to a variety of odorants. Several factors may be responsible for this behaviour, which occurred at a slower rate than the observed labelling. The possibility of damage to the transduction mechanism and/or the inhibition of metabolic processes is discussed elsewhere in this thesis (see Section 4.4); additional explanations include the possible occurrence of enhanced mucus secretion\(^1\) and/or the non-specific labelling of various olfactory receptors (Squirrell, 1978).

Finally, it should be noted that the labelling described in this chapter has been assumed to apply to olfactory receptors. However, in many vertebrates, chemical stimuli can be detected in the nasal cavity via the olfactory system, the vomeronasal system, the trigeminal system\(^2\), the septal organ and possibly the nervus terminalis (Silver and Maruniak, 1981). Although some researchers have cautioned that interaction may occur between these chemoreceptive systems, relatively little has been done to study, or control for, such events; "it generally has been assumed that when one chemoreceptive system is being studied, it alone is responsible for the responses being monitored" (Silver and Maruniak, 1981).

Trigeminal chemoreception has long been considered part of the common chemical sense, functioning to detect noxious chemical stimuli in the mouth, nose and eyes, and to initiate appropriate protective reflexes. However, psychophysical (e.g. see Cain, 1974, 1976; Doty, 1975, Doty et al., 1978; Cain and Murphy, 1980) and electrophysiological (e.g. see Beidler and Tucker, 1956, Tucker, 1963b, 1971;

---

\(^1\) This might be expected since the compounds used as vapour-phase labelling odorants also possess lachrymatory properties.

\(^2\) For a review of trigeminal chemoreception in the nasal and oral cavities, see Silver and Maruniak (1981).
also see Silver and Moulton, 1982; Silver and Mason, 1984) data have recently prompted Silver and Maruniak (1981) to argue that trigeminal chemoreceptors may be stimulated by a wider range of compounds and concentrations than is generally believed\(^1\), such that, "to date there is not sufficient documentation to call any chemical a pure olfactory stimulus". Electrophysiologically, no odorant has yet been tested which does not stimulate nasal trigeminal receptors, although Silver and Moulton (1982) have recently shown that while some compounds (e.g. phenyl ethyl alcohol, benzyl acetate and α-terpinol) stimulated such receptors in some rats, they did not elicit responses in all animals tested, even at vapour saturation.

Some knowledge is now available concerning the types of chemicals that will be potent trigeminal stimuli; most obvious are those causing nasal irritation, pain and physiological reflexes. However, it should be noted that volatile chemicals need not possess an odour to humans to be powerful trigeminal stimulants. The presence of carbon-carbon double bonds, carbonyl and halogen groups (e.g. vapour-phase labelling odorants) and large dipole moments tends to increase the effectiveness of molecules as trigeminal stimuli (e.g. see Doty et al, 1978); the common denominator for trigeminal irritation appears to be an affinity for reacting with sulphydryl groups on proteins (see Silver and Maruniak, 1981).

It is therefore possible that the labelling patterns obtained in this study reflect, at least partially, the behaviour of trigeminal receptors. Since the separation of olfactory and trigeminal components is a major problem in the recording of odorant evoked

\(^1\) At present, it is still unclear what powers of chemical discrimination the trigeminal system may possess (Silver and Maruniak, 1981).
potentials, future studies may circumvent this difficulty by combining electrophysiological investigations on the action potentials recorded from single olfactory neurones (e.g. see Delaleu and Holley, 1980) with biochemical approaches to receptor identification and isolation (e.g. see Chapter 6).

1 It has been suggested that trigeminal and olfactory components of the odorant evoked potential have different latencies and furthermore can be separated on the basis of electrode placement (see Silver and Maruniak, 1981).
CHAPTER 4: VERIFICATION OF VAPOUR-PHASE LABELLING EXPERIMENTS ON THE IN VITRO RAT PREPARATION

4.1 Introduction

Vapour-phase labelling experiments which aim to specifically abolish the EOG responses to a particular odorant are readily open to misinterpretation; the noted absence of signal must be shown to result directly from genuine labelling and not as a consequence of some other action. To this end, several potential sources of error require examination:

(a) The longevity of the employed tissue preparation is of crucial importance, since stable EOG responses to a wide range of odorants must be obtainable over a sufficiently long time span (i.e. hours not minutes) for labelling to occur. Thus, the duration of EOG stability needs to be examined for each type of animal preparation employed. If there should be a decrease with time in the amplitudes of the recorded EOG responses, then such changes should uniformly affect all odorants; there must be no observable differential rate of decrease. Previous studies employing female rat pups (1 - 30 days post partum) have indicated that in response to a test stimulus (ethyl n-butyrate; 10μM; 2 sec. duration) delivered every three minutes, the EOG amplitude declined by 50% in an average of 90 min; no statistically significant correlation between animal age and preparation longevity was evidenced (Gesteland and Sigwart, 1977).

(b) Brominated labelling reagents may undergo hydrolysis (see Figure 4.1) upon contact with the nasal mucus to liberate hydrogen bromide. Consequently, any analysis of the rationale underlying such labelling studies should include an examination of the effect of this compound on the EOG responses obtained to a variety of odorants. Again, the integrity of this chemical modification approach depends upon the
The hydrolysis of halomethyl esters has been found to possess more or less $S_N^2$ character. Thus, the mechanism can be written in the form:

$$\text{RCOOCH}_2\text{X} + \text{H}_2\text{O} \xrightarrow{\text{slow}} \text{RCOOCH}_2\text{OH} + \text{HX}$$

$$\xrightarrow{\text{fast}} \text{RCOOH} + \text{CH}_2\text{O} + \text{HX}$$

(see Duranto, 1969)
absence of any differential rate of EOG abolition in response to either hydrogen bromide or solutions of bromine. Although previous work has alluded to these problems (Squirrell, 1978), little direct experimental data has hitherto been provided.

(c) Two other possible sources of error are inherent in the basic methodology of in vitro vapour-phase labelling experiments (see Chapter 3): (i) The rat preparations are superfused with Ringer solution prior to the recording of EOG responses (see Section 2.3). Thus it is necessary to check that such superfusion does not in itself exert any differential effects upon the EOG responses subsequently obtained. (ii) Furthermore, the reductions noted in the presence of an appropriate labelling reagent must be shown not to be potentiated or exclusively caused by the equivalent non-reactive analogue or other odorants. The repeated pulsing of a particular odorant could produce alterations in the olfactory system (e.g. enhanced mucus secretion; damage to the transduction mechanism or inhibition of metabolic processes (Squirrell, 1978)), resulting in the generation of EOG responses with diminished peak amplitudes. If such diminutions were specific in nature, then a type of "pseudo labelling" would be achieved.

At the commencement of the electrophysiological studies described in this thesis (see Chapter 3), little work had been performed on the in vitro rat preparation; most physiological data had come from frogs (Gesteland and Sigwart, 1977). Consequently the feasibility of using rats in vapour-phase labelling studies required investigation. This

1 Bromine oxidises water, though less readily than chlorine, to produce hypobromous acid and hydrogen bromide:

\[
\text{H}_2\text{O} + \text{Br}_2 \rightarrow \text{HOBr} + \text{HBr}
\]

Exposure of the rat olfactory epithelium to solutions of bromine represents a form of treatment which is potentially more extreme than presentations of hydrogen bromide vapour.
chapter details the innovative work that was performed on such in vitro preparations in order to validate the labelling experiments previously described (see Chapter 3).
4.2 Methods

Studies on the in vitro rat preparation were performed as described elsewhere in this thesis (see Section 3.2(1)). Odorous vapours were applied to the rat olfactory epithelium as previously outlined (see Section 2.4(ii)); bromine (in Ringer solution) and Ringer solutions were administered via glass hypodermic syringes and removed by aspiration.

The application and withdrawal of test solutions did not necessitate removal of the reference electrode from the rat preparation. However, the recording electrode, which was housed in micromanipulators, was temporarily raised during these operations and subsequently lowered such that further EOG responses could be recorded from the original position. Where necessary, solutions applied to the olfactory epithelium were replenished with fresh additions at one minute intervals.

At the outset of every experiment several (at least 3) reproducible (i.e. variation $\leq 2\%$) EOG responses were recorded to each odorant under examination; these peak amplitudes were averaged to give a measure of the baseline response (i.e. 100% value on the accompanying graphs). Test substances, either in the vapour or liquid phase, were then applied to the olfactory epithelium.

The complete removal of all test solutions from the sensory tissue was essential for obtaining good quality EOG responses. After treatment (20 second aliquots) with solutions of bromine, the olfactory epithelium was superfused (5 minutes) with Ringer solution; the tissue was then allowed to rest (5 minutes) before EOG recording was re-commenced.

All EOG responses were recorded in at least duplicate prior to further treatment of the olfactory tissue; these responses were averaged to give the percentage EOG response with respect to the appropriate
baseline value (i.e. % of original EOG response" on the accompanying graphs).

Only rat preparations which gave stable EOG responses during the baseline value determination were used in these experiments. As noted elsewhere (see Section 3.2(i)), the recorded EOG peak amplitudes usually increased with time until stability was attained at about 20 minutes after death.
4.3 Results

The following results show the mean and standard error of the data accumulated from single experiments on separate animals; the number of animals (n) used in each study is indicated.

4.3(i) Stability of the in vitro rat preparation

The longevity of the in vitro rat preparation was examined by recording at regular intervals the EOG responses obtained to various odorants. A typical time course relating the EOG peak amplitudes thus obtained to the life time of such a preparation is shown in Figure 4.2. The origin on the abscissa indicates the time at which decapitation of the rat occurred. Similar time courses were obtained with other odorants. The findings indicate that stable EOG responses could be recorded for up to 4 - 5 hours after the death of the animal.

4.3(ii) Effect on EOG responses of superfusing the rat olfactory epithelium with Ringer solution

The effect of Ringer solution on the EOGs obtained to several odorants was examined. The results obtained, of which Figure 4.3 is representative, show that no diminution of these responses was detected after treatment for the period indicated. Consequently, superfusion (for about 5 minutes; see Section 2.3) of the olfactory epithelium at the commencement of each labelling experiment appears to be acceptable.

4.3(iii) Effects of hydrogen bromide and solutions of bromine on EOG responses obtained from the in vitro rat preparation

Brominated labelling reagents/odorants may undergo hydrolysis upon contact with the nasal mucus to liberate hydrogen bromide. Consequently, the effects of this compound (vapour-phase) and of bromine solutions (see Section 4.1) on the EOGs obtained to a variety of odorants were examined. Figures 4.4 and 4.5 show that each of the reagents brought about a rapid, irreversible abolition of all such
Figure 4.2 Stability of EOG responses obtained from the in vitro rat preparation

EOG signals were obtained at regular intervals from the in vitro rat preparation in response to 2 sec. odour pulses of acetophenone and of 2-isopropyl-3-methoxypyrazine. These results, which were obtained from a single experiment and are representative of 5 further studies, show that EOG stability was maintained throughout the course of the investigation. The abscissa indicates the age of the in vitro preparation, with the origin signifying the time at which decapitation of the rat occurred. EOG responses were recorded every minute up to 120 min, and then at 2 min. intervals. Similar time courses have been obtained with 1,8-cinole; cyclododecyl propionate; ethyl 2-pyrazinocarboxylate; iscamyl acetate; propiophenone, and 3-pyridinmethanol propionate.
Control experiments for vapour-phase labelling studies on the in vitro rat preparation

These figures are presented in the form shown below. The length of each period of exposure of the olfactory epithelium to any test substance may be estimated by reference to the intervals between successive experimental points (here indicated by the letters a - e).

The recovery time permitted after exposing the olfactory epithelium either to hydrogen bromide vapour (see Figure 4.4) or to solutions of bromine (see Figure 4.5) prior to the recording of further EOG responses was varied according to the duration of such treatment, as described in Chapter 3. A 5 min. rest period always followed each aliquot of superfusion with Ringer solution alone (see Figure 4.3). Subsequent to its prolonged stimulation with non-reactive odorants, the olfactory epithelium was allowed to recover as follows:

<table>
<thead>
<tr>
<th>Pulse length of non-reactive odorant (min.)</th>
<th>Recovery time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>1.0</td>
<td>8</td>
</tr>
<tr>
<td>1.5</td>
<td>10</td>
</tr>
<tr>
<td>2.0</td>
<td>12</td>
</tr>
<tr>
<td>3.0</td>
<td>15</td>
</tr>
<tr>
<td>4.0</td>
<td>18</td>
</tr>
<tr>
<td>5.0</td>
<td>20</td>
</tr>
</tbody>
</table>
Figure 4.3  Effect on EOG responses of superfusing the rat olfactory epithelium with Ringer solution

EOG peak amplitude (% of original mean value)

- acetophenone
- 2-isopropyl-3-methoxypyrazine
- 1,8-cineole
- cyclododecyl propionate  n = 5

Cumulative time of superfusion (min)

Similar profiles have been obtained with isoamyl acetate, propiophenone, and 3-pyridinemethanol propionate.

Figure 4.4  Effect of hydrogen bromide vapour on EOG responses obtained from the in vitro rat preparation

EOG peak amplitude (% of original mean value)

- isoamyl acetate
- 1,8-cineole
- 2-isopropyl-3-methoxypyrazine  n = 6

Cumulative time of exposure to hydrogen bromide vapour (0.43 µM) or humidified air (min)

Similar profiles have been obtained with acetophenone, cyclododecyl propionate, ethyl 2-pyrazine-carboxylate, propiophenone and 3-pyridinemethanol propionate.

The lower three traces show the effect of hydrogen bromide vapour on the EOG peak amplitudes obtained to isoamyl acetate, 1,8-cineole and 2-isopropyl-3-methoxypyrazine, whilst the upper three traces represent control experiments in which humidified air (presented to the epithelium via a U-tube containing only liquid paraffin, and employing the same channel of the odour applicator as used for the halogenated vapour) was substituted for the hydrogen
The lower three traces in each of (a), (b) and (c) show the effect on EOG peak amplitudes of superfusing the rat olfactory epithelium with (a) 51 μM, (b) 28 μM and (c) 5 μM solutions of bromine, whilst the upper three traces in each of (a), (b) and (c) represent control experiments in which Ringer solution was substituted for the solutions of bromine. Similar profiles have been obtained with acetophenone, cyclododecyl propionate, ethyl 2-pyrazine-carboxylate, propiophenone and 3-pyridinemethanol propionate.
responses. Moreover, the rapidity of this abolition was influenced by the concentration of reagent which contacted the epithelium (Table 4.1). The accompanying results are representative of similar findings with several other odorants. It is important to note, however, that the EOG responses were never reduced differentially; a uniformity of inhibition was always observed for the range of odorants examined. Consequently the possible hydrolysis of putative labelling reagents does not in itself appear to preclude the performance of valid vapour-phase labelling experiments.

4.3(iv) Effects of prolonged odorant stimulation on EOG responses obtained from the in vitro rat preparation

"Pseudo labelling" experiments, in which non-reactive odorants were employed as vapour-phase labelling reagents in otherwise normal labelling experiments, were performed as previously described (see Section 3.2(i)). The accompanying results show that relatively long pulses (i.e. of similar duration to those used in the application of true labelling reagents) of iscamyl acetate, 3-pyridinemethanol propionate and 2-isopropyl-3-methoxypyrazine brought about a gradual irreversible abolition of the EOG responses obtained to a variety of odorants (Figures 4.6(a) - (c)). This abolition was always non-specific in nature. However, studies which employed cyclododecyl propionate as the non-reactive labelling reagent exhibited differential rates of EOG inhibition (Figure 4.6(d)); after a four minute period (cumulative time) of "labelling" with cyclododecyl propionate, a definite rank order of EOG inhibition could be observed (Table 4.2). Such differential effects highlight possible restrictions in the performance of labelling experiments with high molecular weight reactive odorants.
Table 4.1  Effects on EOG responses of superfusing the rat olfactory epithelium with different concentrations of bromine solution

<table>
<thead>
<tr>
<th>Concentration of bromine solution (µM)</th>
<th>Odorant</th>
<th>EOG peak amplitude (% of original mean value) after 40 seconds (cumulative time) labelling (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1,8-Cineole</td>
<td></td>
</tr>
<tr>
<td>51 (n = 5)</td>
<td>5 ± 2</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>28 (n = 5)</td>
<td>8 ± 3</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>5 (n = 5)</td>
<td>25 ± 5</td>
<td>31 ± 3</td>
</tr>
<tr>
<td></td>
<td>2-Isopropyl-3-methoxypyrazine</td>
<td>6 ± 2</td>
</tr>
<tr>
<td></td>
<td>12 ± 4</td>
<td>38 ± 5</td>
</tr>
</tbody>
</table>

Tabular representation of some of the data presented in Figure 4.5 showing the mean ± S.E.M. values of the EOG peak amplitudes (expressed as a % of the original mean baseline response) obtained after superfusing the rat olfactory epithelium with each of three different concentrations of bromine solution for 40 seconds (cumulative time).
Profiles similar to those shown in (a) - (c) (i.e., eventual non-specific reductions of all EOG responses) have been obtained following prolonged stimulation of the rat olfactory epithelium with each of the odorants employed in the vapor-phase labelling experiments described previously (see Chapter 3).
Table 4.2  Effect of prolonged stimulation with cyclododecylpropionate on EOG responses obtained from the in vitro rat preparation

<table>
<thead>
<tr>
<th>Odorant</th>
<th>EOG peak amplitude (% of original mean value) after 4 minutes (cumulative time) labelling (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoamyl acetate</td>
<td>53 ± 3</td>
</tr>
<tr>
<td>3-Pyridinemethanol propionate</td>
<td>50 ± 5</td>
</tr>
<tr>
<td>2-Isopropyl-3-methoxypyrazine</td>
<td>41 ± 5</td>
</tr>
<tr>
<td>Cyclododecyl propionate</td>
<td>7 ± 3</td>
</tr>
</tbody>
</table>

Tabular representation of some of the data presented in Figure 4.6(d) showing the mean ± S.E.M. values of the EOG peak amplitudes (expressed as a % of the original mean baseline response) obtained after exposing the rat olfactory epithelium to cyclododecyl propionate (0.44 μM) for 4 minutes (cumulative time) (n = 8)
4.4 Discussion

The electrophysiological stability exhibited by the in vitro rat preparation was comparable with previous findings on the frog and on sheep (e.g. see Menevse, 1977; Menevse et al, 1978; Squirrell, 1978; Poynder et al, 1978; also see Gesteland and Sigwart, 1977). Consequently, it appears reasonable to regard the life span of this preparation as being adequate for the performance of vapour-phase labelling experiments.

Hydrogen bromide and solutions of bromine rapidly abolished the EOG responses obtained to a variety of odorants. However, neither of these compounds, across the concentration range examined, ever gave rise to differential EOG inhibition. This absence of selectivity supports the employment of bromoalkyl esters as potential labelling reagents.

The vapour-phase experiments previously described (see Chapter 3) effectively eliminate the possibility of any problems arising from hydrolysis of the reactive odorants. In these studies, labelling molecules in the mucus are continuously being replaced by molecules from the vapour-phase. Consequently, hydrolysis must occur at a faster rate than this replenishment process if it is to exert any significant effect; the rate of hydrolytic decomposition of bromoacetic acid has been found to be 0.20 hour⁻¹ (pH 7.0; 37°C) (Barnard and Stein, 1959). In addition, Squirrell (1978) reported that ethyl bromoacetate exhibited no decomposition products (as analysed by gas-liquid chromatography) after being shaken for

---

¹ The time taken for odorant molecules to reach the appropriate receptor sites must be less than the period of EOG latency; the latency of response to stimulation with butanol has been reported to vary from 0.2 to 0.4 seconds for the frog (Ottoson, 1956).
30 minutes with an excess of frog's Ringer solution.

The substitution of non-reactive analogues for vapour-phase labelling odorants in otherwise normal labelling experiments led to a gradual reduction in the EOG responses obtained to a variety of odorants; isoamyl acetate, 3-pyridinemethanol propionate and 2-isopropyl-3-methoxypyrazine all gave rise to non-specific diminutions, whereas cyclododecyl propionate exhibited a differential rate of EOG inhibition.

The reduction in EOG responses brought about by prolonged odorant stimulation of the olfactory epithelium is particularly important: it places constraints upon the choice and deployment of potentially useful vapour-phase labelling reagents. Similar observations on the responses of single receptor neurones in the young rat have been previously reported: such responses were repeatable for at least 30 minutes provided intense stimulation was avoided, whereupon the number of evoked spikes diminished with each successive stimulus presentation until all responsiveness was lost after 5 - 10 stimulus events (Gesteland and Sigwart, 1977).

None of the odorants employed in the present labelling experiments (see Chapter 3) ever produced differential rates of EOG inhibition; this discredits the thesis that the selectivity observed is induced by the odorants themselves and does not reflect specific inactivation by the vapour-phase labelling reagents.

Factors which could account for the observed non-specific reductions of EOG responses include possible damage to the transduction mechanism and/or the inhibition of metabolic processes. Although the

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1 E.g. the previously determined stability of the in vitro rat preparation (i.e. 4 - 5 hours) may be detrimentally affected by the routine application of long odorant pulses to the olfactory epithelium.
latter may be expected to occur (Webb, 1966), it has been shown that inhibitors of glycolysis or of oxidative phosphorylation do not immediately affect EOG production (Getchell, 1971). However, inhibition of the electrogenic sodium pump may explain some of the observed reductions; ouabain (an inhibitor of Na⁺-K⁺-ATPase activity) has been reported to cause the diminution of EOG responses, albeit "much more slowly" than appropriate group specific reagents (Getchell, 1971).

The differential inhibition of EOG responses brought about by cyclododecyl propionate resembles genuine vapour-phase labelling. Consequently, studies employing a reactive derivative of this odorant would seem likely to yield artifactual data. At present, the basis of this selective behaviour remains unclear, although the previously discussed factors (i.e. damage to the transduction mechanism; inhibition of metabolic processes) may again be involved.

The differential effect produced by cyclododecyl propionate appears consistent with the phenomenon of selective adaptation (fatigue), whereby the sensitivity of the olfactory epithelium to particular substances is selectively reduced¹ (Ottoson, 1956). However, this in itself cannot account for the observed differential inhibition of EOG responses which was found to be irreversible with time.

¹ This behaviour has been interpreted as evidence for the existence of "receptors which are specially sensitive to certain substances or to certain molecular characteristics of the stimulating agents" (Ottoson, 1956).
CHAPTER 5: THE EFFECT OF CONCANAVALIN A ON FROG OLFACTORY MUCOSA

5.1 Introduction

5.1(i) Group-specific reagents

These reagents exhibit specificity only with respect to their chemical reactivity. Thus, a variety of "thiol reagents" react only with cysteine sidechains; various alkylating reagents react with several nucleophilic amino acid sidechains (including serine, lysine and cysteine) and tetranitromethane specifically labels the phenolic side chain in tyrosine residues. Such group-specific labelling (see Figure 5.1; also see Figure 3.1 (affinity labelling)) has been reviewed generally by Singer (1967) and more particularly with respect to the identification of olfactory receptors by Todd and Persaud (1981).

The first chemical modification studies performed on the vertebrate olfactory system utilised the group-specific reagent, N-ethyl maleimide (NEM) (Getchell, 1971; Getchell and Gesteland, 1972). This "thiol reagent", when applied in solution to the frog olfactory epithelium, irreversibly decreased (by 77%) the EOG responses obtained to the fruity odorant, ethyl butyrate. Protection experiments using solutions of the odorant showed that this inhibition could be prevented.

Getchell and Gesteland (1972) interpreted their findings as evidence for the existence of specialised proteinaceous olfactory receptor sites. However, it should be noted that two features of this pioneering study complicate interpretation of the data. Since NEM is membrane-permeable (Vignais and Vignais, 1973), the observed inhibition could have been due to reaction with an intracellular protein involved in ion conductance rather than with an extracellular...
Figure 5.1  Schematic representation of group-specific labelling

\[ L + R \rightarrow L-R \]

L = chemical modification reagent
R = receptor
receptor protein. Secondly, high concentrations \((10^{-2} \text{ M})\) of the odorant were required for protection; at these concentrations, a proportion of the lipophilic odorant may have permeated the plasma membrane.

The thiol groups involved in the frog olfactory response were further investigated using the non-permeable thiol reagent, mersalyl\(^1\) and the fruity odorant, pentyl acetate (Menevse, 1977; Menevse et al, 1978). Protection by solutions of this odorant was observed; the response to some odours was protected to a greater extent than to others (see Table 5.1). This differential protection can be interpreted as providing evidence for the existence of classes of olfactory receptor proteins.

Recent studies have examined the effects of mersalyl and enzymatic iodination (specific for tyrosine residues) on the EOG responses obtained from the rat (Shirley et al, 1980; 1981; 1983a). The findings were consistent with an olfactory mechanism involving relatively non-specific receptor proteins; each receptor type was envisaged as interacting weakly with a number of odorants and each odorant with a number of receptors.

The vapour-phase modification of frog olfactory receptor proteins has been achieved with several group-specific reagents (Menevse et al, 1977b). The thiol reagent, 4-chloro-7-nitrobenzofurazan (Nbf-chloride) (see Gishah and Whitehouse, 1968; Fagar et al, 1973) was found to preferentially inhibit the EOG responses to itself; much smaller decreases were observed in the EOG responses to 1,8-cineole and to pentyl acetate (Figure 5.2). N-Ethyl maleimide (NEM) (see Delaleu and Holley, 1980),

\[^1\text{(3-[[2-(carboxymethoxy)-benzoyl] amino]-2-methoxypropyl) hydroxymercury monosodium salt }^\text{see Xavier and Hanoune, 1975).\]
Table 5.1: Ability of pentyl acetate to protect odorant-binding sites against labelling by the thiol-specific reagent mersalyl

<table>
<thead>
<tr>
<th>Odorant</th>
<th>Odour description</th>
<th>E.O.G. peak amplitude (% of original)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentyl acetate</td>
<td>Fresh, fruity, pears</td>
<td>86 ± 4 (24)</td>
</tr>
<tr>
<td>n-Butyl acetate</td>
<td>Ethereal, fruity, pungent</td>
<td>78 ± 7 (2)</td>
</tr>
<tr>
<td>Cinneole</td>
<td>Camphoraceous</td>
<td>77 ± 11 (3)</td>
</tr>
<tr>
<td>n-Butyric acid</td>
<td>Sour, reminiscent of rancid butter</td>
<td>74 ± 10 (3)</td>
</tr>
<tr>
<td>Phenacetalddehyde</td>
<td>Green, floral, sweet, hyacinth</td>
<td>72 ± 2 (2)</td>
</tr>
<tr>
<td>Butan-1-ol</td>
<td>Fusel oil, winey</td>
<td>50 ± 6 (2)</td>
</tr>
<tr>
<td>Nitrobenzene</td>
<td>Pungent, bitter almond</td>
<td>48 ± 10 (3)</td>
</tr>
<tr>
<td>Beazyl acetate</td>
<td>Sweet, floral, fresh, fruity, jasmine</td>
<td>38 ± 10 (2)</td>
</tr>
<tr>
<td>β-Ionone</td>
<td>Warm, woody, dry, fruit undertone, violets</td>
<td>35 ± 8 (4)</td>
</tr>
<tr>
<td>Linalyl acetate</td>
<td>Sweet, floral, fruity, recalling bergamot and pears</td>
<td>28 ± 11 (2)</td>
</tr>
</tbody>
</table>

The results are expressed as the percentage of the original E.O.G. peak amplitude before any reagents were applied; they show the values (means ± S.E.M. for the numbers of experiments given in parentheses) obtained 34 min. after washing the frog olfactory mucosa with Ringer solution following treatment of the mucosa with mersalyl (0.1 mM) and pentyl acetate (1 mM).

Taken from Menevse et al (1978)
Figure 5.2 Specific labelling of receptors for the odorant 4-chloro-7-nitrobenzofurazon

(a) Typical electro-olfactogram for this odorant; (b) typical electro-olfactogram for n-amyl acetate. The points in (c) are means ± S.E.M. of three experiments. □, 1,8-Cinole; ○, n-amyl acetate; Δ, 4-chloro-7-nitrobenzofurazan. The vapour pulses were of 10s duration, with an interval of 2 min between each. The general experimental methods were as described by Getchell & Gesteland (1972).

Taken from Henevse et al. (1977b)
1-fluoro-2,4-dinitrobenzene and benzyl chloride have also given rise to specific, vapour-phase chemical modification. Selectivity of labelling was not observed when these reagents were applied to the olfactory epithelium in the liquid phase.

5.1(ii) Concanavalin A

The first agglutinin to be isolated in relatively pure form was a crystallised lectin from the jack bean (Canavalia ensiformis). This particular fraction, which was initially referred to as concanavalin fraction A, later became known as concanavalin A or Con A (see Sumner and Howell, 1936).

The importance of this globular lectin has been emphasised in recent years by the discovery of numerous interesting properties: it forms insoluble complexes with many polysaccharides (Goldstein et al, 1965) and glycoproteins (Clarke and Denborough, 1971); it is a potent mitogen (Douglas et al, 1969; Novogrodsky and Katchalski; 1971; Powell and Leon, 1970) which stimulates the blast formation of lymphocytes (Ferlmann et al, 1970); it inhibits the migration of tumour cells in in vitro chambers (Friberg et al, 1972) and low dosages of Con A have been found to be immunosuppressive in some skin allograft experiments (Markowitz et al, 1969; also see Toledo-Pereyra et al, 1975). In addition, Con A has been reported to inhibit fertilisation (Lallier, 1972), phagocytic activity (Allan et al, 1971) and tumour cell growth (Burger and Noonan, 1970; Shoham et al, 1970).

1 For a discussion of various physicochemical and biological properties of concanavalin A, see Chowdhury and Weiss (1975); Bittiger and Schnebli (1976).
Lectins, and in particular concanavalin A, have been extensively used as probes for membrane surfaces\(^1\) (see Bittiger and Schnebli, 1976); in some instances, the specific inactivation of receptors (comparable to that obtained by chemical modification) has been achieved. This suggested that lectins may permit the identification of different types of olfactory receptors by differentially inhibiting their activity to various classes of odorants. Consequently, the effects of concanavalin A on vertebrate olfactory responses were examined. The studies described in this chapter outline the effect of Con A on frog olfactory mucosa; they were performed in parallel with similar and more exhaustive investigations on the in vitro rat preparation (Polak et al., 1982; Polak, 1983; Shirley et al., 1983b, 1986; see Section 5.3(i)).

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\(^1\) Recent studies have assessed whether plant lectins exhibit any preferential or specific binding to the various cell types present in the rat olfactory epithelium, since these molecules offer the potential of ideal purification probes (Hempstead and Morgan, 1983). Fluorescein-conjugated peanut lectin was found to selectively stain duct- and gland-like structures in both the mucosa and lamina propria mucosae with no background staining, whilst concanavalin A and fluorescent pokeweed mitogen differentially stained the mature olfactory receptor neurones and the brush border of the neuroepithelium respectively, albeit with a general global background staining of lesser intensity. Since Con A binds predominantly to mannosyl residues (Goldstein and So, 1965), "it might be inferred that the mature receptor neurones have a greater abundance of this sugar in their membranes than do their precursor neuroblasts in the basal cell layer" (Hempstead and Morgan, 1983; also see Section 5.4).
5.2 Methods

The animal (rat and frog) preparations and methodology employed have been generally described elsewhere (see Sections 2.2; 2.3; 3.2(i); 4.2). Concanavalin A (or pyridoxal phosphate; see Section 5.3(iii)) was dissolved in mammalian or frog's Ringer solution (as appropriate) and applied to the olfactory epithelium with a Pasteur pipette.

The standard protocol involved the recording of several (at least three) reproducible (i.e. variation $\leq \pm 2\%$) EOG responses to each of the odorants under investigation. The mean of these EOG peak amplitudes represented the "100% value" (i.e. baseline response) for each compound. Modification experiments were performed only on preparations which exhibited stable responses. Having slightly raised the recording electrode, the preparation was (su)perfused initially with the reagent solution$^1$ and then with Ringer solution alone for about 5 minutes. Following a rest period (10 minutes for the frog preparations; 2 minutes for the rat preparations), the measuring electrode was replaced and EOG responses were again recorded until at least three identical signals were obtained to each odorant. This procedure was repeated if further treatment was desired.

The odorants under investigation were applied to the olfactory mucosa in a predetermined sequence; isoamyl acetate, which was always employed as a reference odorant, constituted every fourth odour pulse. EOG peak amplitudes were normalised with respect to this standard odorant. The order of odorant delivery was randomised for replicate experiments. Frog preparations were stimulated with 2 second odour pulses, followed by 2 minute pulse intervals to prevent adaptation.

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$^1$ For the control experiments, Ringer solution was used in place of the modifying reagent.
(see van Boxtel and Koster, 1978); shorter odour pulses (1 second) were applied to the in vitro preparations and EOG responses were recorded every minute.

The in vitro rat preparations generally attained stability more quickly than the in vivo frog preparations, both before and after treatment with the modifying reagent; reproducible EOG responses could be recorded from the frog only after periodic drainage of the nasal cavity for anything up to one hour.

The stability of the preparations towards isoamyl acetate was indicated by the parameter, \( L \), where

\[
L = \frac{A_f}{A_i}
\]

\( A_i \) represents the mean amplitude of the EOG responses obtained to isoamyl acetate before treatment with the modifying reagent, and \( A_f \) signifies that obtained after treatment. The effect of concanavalin A (or pyridoxal phosphate; see Section 5.3(iii)) is described by the response parameter, \( R \), which denotes the ratio of the normalised EOG amplitudes obtained before and after treatment. Thus, an \( R \) value of \(<1\) indicates that the response to this particular odour is affected to a greater extent than the response to the standard odorant. All the preparations used in these present studies exhibited high values of \( L (\geq 95\%) \). Consequently, \( R \) is a measure of the effect of the modifying reagent on a particular set of receptor cells (see Shirley et al., 1983b; 1986).

\[
R = \frac{\text{EOG}_f(\text{odorant})}{\text{EOG}_f(\text{isoamyl acetate})} \frac{\text{EOG}_i(\text{isoamyl acetate})}{\text{EOG}_i(\text{odorant})}
\]

where \( \text{EOG}_i(\text{odorant}) \) and \( \text{EOG}_i(\text{isoamyl acetate}) \) respectively represent the mean amplitudes of the EOG responses obtained to a particular odorant and to isoamyl acetate before treatment with the modifying reagent, and \( \text{EOG}_f(\text{odorant}) \) and \( \text{EOG}_f(\text{isoamyl acetate}) \) signify those obtained after treatment.
5.3 Results

The accompanying results show the values obtained for the mean normalised EOG amplitudes \( R \) together with their 95% confidence intervals; each value of mean \( R \) was determined from experimentation on at least 3 animal preparations.

5.3(i) Superfusion of rat olfactory mucosa with concanavalin A

Concurrent studies on the in vitro rat preparation showed that concanavalin A could differentially inhibit the EOGs obtained to a variety of odorants; the responses to \( C_4 - C_6 \) alkanoic and alicyclic carboxylic acids (and possibly to aliphatic thiols) were preferentially reduced (Polak et al, 1982; Polak, 1983; Shirley et al, 1983b; 1986).

In order to utilise these findings as reference points for the in vivo studies on the frog, the present work initially sought their reproduction and expansion. Consequently, the effect of Con A (0.5 mg/ml) on the EOG responses obtained to several odorants (including heterocycles) was examined.

The results (Table 5.2) show that the EOG peak amplitudes were differentially affected by concanavalin A: the responses to isovaleric acid\(^1\), nicotinic acid, 2-pyrazinecarboxylic acid and 3-pyridylacetic acid were more inhibited than those obtained to 3-pyridylacetonitrile, \( n \)-propanol\(^1\) and trimethylamine. 1,8-Cineole\(^1\) exhibited somewhat intermediate behaviour. These findings, which are consistent with those obtained from the parallel investigations (see Shirley et al, 1983b; 1986), suggested that a similar experimental protocol could now be applied to the in vivo frog preparation.

\(^1\) The \( R \) values obtained for these compounds are very similar to those reported by Shirley et al (1986).
Table 5.2 Effect of concanavalin A (0.5 mg/ml) on EOG responses obtained from the in vitro rat preparation

<table>
<thead>
<tr>
<th>Odorant</th>
<th>M.Wt.</th>
<th>With Con A (5 min)</th>
<th>Without Con A (5 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isovaleric acid</td>
<td>102</td>
<td>0.36 ± 0.14</td>
<td>0.93 ± 0.06</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>123</td>
<td>0.47 ± 0.14</td>
<td>0.94 ± 0.10</td>
</tr>
<tr>
<td>2-Pyrazine-carboxylic acid</td>
<td>124</td>
<td>0.48 ± 0.12</td>
<td>0.92 ± 0.07</td>
</tr>
<tr>
<td>3-Pyridylacetic acid</td>
<td>137</td>
<td>0.65 ± 0.21</td>
<td>1.05 ± 0.15</td>
</tr>
<tr>
<td>1,8-Cineole</td>
<td>154</td>
<td>0.79 ± 0.13</td>
<td>0.98 ± 0.08</td>
</tr>
<tr>
<td>3-Pyridylacetocnitrile</td>
<td>118</td>
<td>0.84 ± 0.12</td>
<td>0.94 ± 0.09</td>
</tr>
<tr>
<td>n-Propanol</td>
<td>60</td>
<td>0.89 ± 0.14</td>
<td>0.96 ± 0.08</td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>59</td>
<td>0.01 ± 0.08</td>
<td>0.99 ± 0.06</td>
</tr>
</tbody>
</table>

The olfactory epithelium was superfused with either mammalian Ringer solution or Ringer solution containing concanavalin A (0.5 mg/ml), as described in Section 5.2. The results, obtained from at least three animal preparations, are expressed as the mean value of the normalised EOG (R) ± 95% confidence intervals.
Perfusion of frog olfactory mucosa with concanavalin A

Preliminary investigations employing concanavalin A at the same concentration as that used successfully with the in vitro rat preparation (i.e. 0.5 mg/ml) produced negligible ECG inhibition, even after 2 x 5 minute periods of exposure to the modifying reagent (Table 5.3). However, differential inhibition was observed when the concentration of Con A was increased by an order of magnitude (Table 5.4). Three levels of effect were noted (Figure 5.3); the ECGs obtained to carboxylic acids were the most affected, with the smaller odorants (i.e. acrylic acid, n-butyric acid, isovaleric acid and cyclopentanecarboxylic acid) exhibiting the more diminished responses. The responses to a wide range of unrelated odorants were considerably less reduced.

It should be noted that two odorants (t-butanethiol and 3-pyridylacetic acid) appear inconsistent with this general pattern of behaviour. At present, it is not possible to readily account for the diminished EOGs obtained to t-butanethiol; such an explanation must await the performance of further studies. However, the comparatively limited inhibition of responses to 3-pyridylacetic acid may reflect the fact that this compound was used in its hydrochloride form.

The EOGs obtained to the various acids were generally smaller (i.e. \( \leq 1 \text{ mV} \)) than those recorded to the other odorants. Base line noise was frequently detected after pulsing with the more potent acidic compounds (e.g. acrylic acid, n-butyric acid); this behaviour precluded extensive investigations with compounds such as acetic acid.

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1 Similar levels of EOG reduction for t-butanethiol (\( R = 0.55 \pm 0.09 \)) have been detected after exposing rat olfactory receptor sites to concanavalin A (0.5 mg/ml) (Shirley et al, 1986).
Table 5.3 Effect of concanavalin A (0.5 mg/ml) on EOG responses obtained from the in vivo frog preparation

<table>
<thead>
<tr>
<th>Odorant</th>
<th>M. Wt.</th>
<th>With Con A (1 x 5 min)</th>
<th>Without Con A (1 x 5 min)</th>
<th>With Con A (2 x 5 min)</th>
<th>Without Con A (2 x 5 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isovaleric acid</td>
<td>102</td>
<td>0.91 ± 0.05</td>
<td>0.93 ± 0.03</td>
<td>0.84 ± 0.06</td>
<td>0.91 ± 0.04</td>
</tr>
<tr>
<td>Cyclopentane-carboxylic acid</td>
<td>114</td>
<td>0.90 ± 0.03</td>
<td>0.93 ± 0.03</td>
<td>0.91 ± 0.03</td>
<td>0.90 ± 0.03</td>
</tr>
<tr>
<td>t-Butanethiol</td>
<td>90</td>
<td>0.98 ± 0.04</td>
<td>0.98 ± 0.03</td>
<td>0.91 ± 0.06</td>
<td>0.96 ± 0.07</td>
</tr>
<tr>
<td>1,8-Cineole</td>
<td>154</td>
<td>0.96 ± 0.04</td>
<td>0.99 ± 0.04</td>
<td>0.94 ± 0.05</td>
<td>0.99 ± 0.03</td>
</tr>
<tr>
<td>m-Cresol</td>
<td>108</td>
<td>0.98 ± 0.06</td>
<td>0.94 ± 0.04</td>
<td>0.97 ± 0.05</td>
<td>0.92 ± 0.05</td>
</tr>
<tr>
<td>2-Isobutyl-3-methoxy-pyrazine</td>
<td>166</td>
<td>1.00 ± 0.02</td>
<td>1.03 ± 0.02</td>
<td>0.99 ± 0.03</td>
<td>1.01 ± 0.04</td>
</tr>
</tbody>
</table>

The olfactory epithelium was perfused with either frog's Ringer solution or Ringer solution containing concanavalin A (0.5 mg/ml), as described in Section 5.2. The results, obtained from at least three animal preparations, show the values of the mean normalised EOG amplitudes (R) ± 95% confidence intervals.
Table 5.4 Effect of concanavalin A (5 mg/ml) on EOG responses obtained from the in vivo frog preparation

<table>
<thead>
<tr>
<th>Odorant</th>
<th>M.Wt.</th>
<th>(R) MEAN ± 95% C.I.</th>
<th>With Con A (5 min)</th>
<th>Without Con A (5 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylic acid</td>
<td>72</td>
<td>0.32 ± 0.13</td>
<td>0.90 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>n-Butyric acid</td>
<td>88</td>
<td>0.33 ± 0.12</td>
<td>0.94 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>Isovaleric acid</td>
<td>102</td>
<td>0.38 ± 0.10</td>
<td>0.91 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>Cyclopentane-carboxylic acid</td>
<td>114</td>
<td>0.41 ± 0.06</td>
<td>0.90 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>123</td>
<td>0.52 ± 0.08</td>
<td>0.95 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>t-Butanethiol</td>
<td>90</td>
<td>0.53 ± 0.08</td>
<td>0.96 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>2-Pyrazine-carboxylic acid</td>
<td>124</td>
<td>0.56 ± 0.09</td>
<td>0.93 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>3-Pyridylacetic acid</td>
<td>137</td>
<td>0.71 ± 0.18</td>
<td>1.03 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>Dimethylethylpyrazine</td>
<td>136</td>
<td>0.79 ± 0.07</td>
<td>0.91 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>3-pyridinemethanol</td>
<td>109</td>
<td>0.80 ± 0.11</td>
<td>0.92 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>1,8-Cineole</td>
<td>154</td>
<td>0.83 ± 0.08</td>
<td>0.99 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>3-Pyridylautonitrile</td>
<td>118</td>
<td>0.88 ± 0.09</td>
<td>0.92 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Acetophenone</td>
<td>120</td>
<td>0.89 ± 0.05</td>
<td>0.97 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>n-Propanol</td>
<td>60</td>
<td>0.91 ± 0.06</td>
<td>0.96 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>m-Cresol</td>
<td>108</td>
<td>0.97 ± 0.09</td>
<td>0.89 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>59</td>
<td>0.97 ± 0.06</td>
<td>1.01 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>2-Isobutyl-3-methoxypyrazine</td>
<td>166</td>
<td>0.99 ± 0.03</td>
<td>1.02 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

The olfactory epithelium was perfused with either frog's Ringer solution or Ringer solution containing concanavalin A (5 mg/ml), as described in Section 5.2. The results show the mean values of the normalised EOG amplitudes (R) together with their 95% confidence intervals; each value of mean R was determined from experimentation on at least four animal preparations.
Diagrammatic representation of some of the data presented in Table 5.4 showing the mean values of the normalised EOG amplitudes (R) ± 95% confidence intervals obtained after perfusing the olfactory epithelium with frog's Ringer solution containing concanavalin A (5 mg/ml).
Similar effects have been observed with the in vitro rat preparation (S. Shirley, personal communication).

In general, the effects of concanavalin A (5 mg/ml) on frog olfactory mucosa were similar to those brought about in the rat by a reduced concentration (0.5 mg/ml) of lectin.
5.3(iii) **Perfusion of frog olfactory mucosa with pyridoxal phosphate**

In a further attempt to bring about selective EOG inhibition, the effect of pyridoxal phosphate (dissolved in frog's Ringer solution at the limit of its solubility, i.e. about 5 mg/ml) on frog olfactory mucosa was examined: studies on the in **vitro** rat preparation have tentatively suggested that this reagent (3 mg/ml Ringer solution) can differentially inhibit the EOGs obtained to a variety of odorants (E. Polak, personal communication).

Table 5.5 shows that negligible EOG inhibition was detected after exposing the olfactory mucosa to pyridoxal phosphate for up to 10 minutes (cumulative time). Since the reagent's limited solubility precluded treatment at higher concentrations, no further attempts were made during the present studies to bring about selective odorant inhibition.
### Table 5.5  
Effect of pyridoxal phosphate (5 mg/ml) on EOG responses obtained from the in vivo frog preparation

<table>
<thead>
<tr>
<th>Odorant</th>
<th>M. Wt.</th>
<th>(R) MEAN ± 95% C.I.</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>With pyridoxal</td>
<td>Without</td>
<td>With pyridoxal</td>
<td>Without</td>
<td>With</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phosphate (1 x 5 min)</td>
<td>pyridoxal</td>
<td>phosphate (2 x 5 min)</td>
<td>pyridoxal</td>
<td>pyridoxal phosphate (1 x 5 min)</td>
</tr>
<tr>
<td>2-Pyrazine-carboxylic</td>
<td>124</td>
<td>0.91 ± 0.05</td>
<td>0.94 ± 0.04</td>
<td>0.85 ± 0.06</td>
<td>0.91 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimethylethyl-pyrazine</td>
<td>136</td>
<td>0.94 ± 0.04</td>
<td>0.96 ± 0.03</td>
<td>0.90 ± 0.05</td>
<td>0.92 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>3-Pyridine methanol</td>
<td>109</td>
<td>0.95 ± 0.06</td>
<td>0.97 ± 0.04</td>
<td>0.92 ± 0.07</td>
<td>0.95 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>1,8-Cineole</td>
<td>154</td>
<td>0.97 ± 0.03</td>
<td>0.99 ± 0.03</td>
<td>0.94 ± 0.05</td>
<td>0.98 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>59</td>
<td>0.99 ± 0.03</td>
<td>1.00 ± 0.02</td>
<td>0.98 ± 0.03</td>
<td>0.99 ± 0.03</td>
<td></td>
</tr>
</tbody>
</table>

The olfactory epithelium was perfused with either frog's Ringer solution or Ringer solution containing pyridoxal phosphate (5 mg/ml), as described in Section 5.2. The results, obtained from at least three animal preparations, are expressed as the mean value of the normalised EOG (R) ± 95% confidence intervals.
5.3(iv) Recovery of EOG responses

Following the (su)perfusion of frog or rat olfactory mucosa with concanavalin A, diminished EOG responses were never observed to recover. Thus, the effects produced by Con A appear to be irreversible.

5.3(v) Appearance of EOG responses

Figure 5.1 shows a sequence of EOG responses obtained during a typical experiment on the in vivo frog preparation. Some signals recorded from the in vitro rat preparation have been presented elsewhere (see Shirley et al., 1986).
Figure 5.4  Some EOG signals from a typical experiment on the in vivo frog preparation

(a)  

These EOG signals were recorded (a) before and (b) after superfusion (5 min) of the frog olfactory epithelium with concanavalin A (5 mg/ml) (see Figure 5.3). The stimulus pulse was 2 sec., sufficient to elicit the EOG peak.
5.4 Discussion

The results show that concanavalin A exerts a marked effect on frog olfactory mucosa; the EOG responses to carboxylic acids are affected differently from those of other odorants examined, with the exception of t-butanethiol.

The interpretation of these findings is critically dependent upon the definition of the response parameter, $R^1$ (see Shirley et al., 1986). If there were a single receptor protein for small carboxylic acid odorants, then $R$ would (as in the case of most chemical modification studies on receptor proteins) measure the extent of protein inactivation. However, this present work shows consistency with the studies performed on the in vitro rat preparation (Shirley et al., 1983b; 1986): $R$ is interpreted in terms of a model which assumes that there are multiple receptor proteins for each class of odorant and that most odorants bind to more than one type of receptor (Polak, 1973; see Shirley et al., 1986). Consequently, it is assumed that concanavalin A may be inhibiting one protein in a set of receptor proteins and that the percentage change in the $R$ value reflects the relative degree of interaction of the odorants with the inhibited protein. Throughout the current work, higher concentrations of Con A (i.e. $>5$ mg/ml) did not reduce further the especially low $R$ values of the appropriate fatty acids.

The present investigations with the frog fulfilled a dual purpose. Firstly, they permitted identification of the effects of concanavalin A on the EOG responses obtained to aliphatic carboxylic acids; volatile fatty acids are effective odorants for all the vertebrates so far examined, and so Con A would be expected to bring

---

1 For a detailed discussion of the interpretation of $R$, see Shirley et al (1986).
about a pattern of selective odorant inhibition in the frog
similar to that found in the rat (Polak et al., 1982; Polak, 1983;
Shirley et al., 1983b; 1986). Since the in vitro studies were more
exhaustive and comprehensive than those presently reported, any
concanavalin A-induced effects detected with the frog may be
confirmed by reference to these parallel investigations.

Secondly, as part of the general search for reagents capable
of specifically affecting olfactory receptor sites directed against
heterocyclic odorants (see Chapter 3), the effects of concanavalin A
on the EOG responses obtained to heterocyclic acids were examined.
However, although the EOGs to nicotinic acid and 2-pyrazinecarboxylic
acid were inhibited by concanavalin A, the reductions (R = 0.52; 0.56
respectively) were not as great as those found with the aliphatic
carboxylic acids (R≤0.4). Consequently, additional studies on a
wider range of heterocyclic acids were neither encouraged nor
performed.

The preferential inhibition of the EOG responses to small,
sweaty-smelling fatty acids may reflect the binding of concanavalin A
to a glycoprotein which is part of, or simply adjacent to, a receptor
protein system for these odorants. The reason for such a Con A-
sensitive protein at this site and not at others is still unclear.
However, the observation of a distinct pattern of differential
inhibition argues against a non-specific interaction of concanavalin A

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1 The preferential inhibition of such responses has also been achieved
with the in vitro rat preparation (R<0.4); this selectivity, which
was consistent with observations on the human sweaty specific
anosmia (e.g. see Amoore, 1967), was regarded as being compatible
with the existence of "a specific receptor system" for sweaty-
smelling fatty acids (Shirley et al., 1986; also see Shirley et al
1983b; but see Boelens et al., 1983). Additional support for such
specificity has been provided by the observation that two strains
of mice, which can smell odorants such as amyl acetate and
pentadecalactone, are insensitive to the sweaty smelling compound
isovaleric acid (see Wysocki, 1984).
with the EOG-generating system. The lack of EOG inhibition in the control experiments is evidence against a pH-effect induced by the acid odorants. Further, the limited inhibition experienced by n-propanol and trimethylamine refutes the thesis that the noted selectivity simply mirrors an effect induced by water-soluble odorants.

The concentration of concanavalin A required to bring about EOG inhibition in the frog is more than two orders of magnitude greater than that required for an effect on many established receptors (Bittiger and Schnebli, 1976), and about one order of magnitude greater than that required to achieve a similar effect in the rat olfactory mucosa (Shirley et al., 1983b, 1986). This may be explained by the binding of Con A to the olfactory mucus; the mucus layer overlying the frog olfactory receptors is much thicker (25 - 30µ; Reese, 1965) than that in the rat and so the higher lectin concentration required may reflect considerable adsorption of the protein to the mucus.

The studies on the in vitro rat preparation have shown that "the reduction of the EOGs to fatty acid odorants brought about by concanavalin A could be prevented by adding 20 mM methyl mannoside."

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1 Studies performed on the rat have also shown that the inhibitory action of concanavalin A is not simply induced by malodours; the lectin exerted little effect on the EOG responses obtained to n-butylisonitrile (R = 0.97 ± 0.09; Shirley et al., 1986).

2 The magnitude of this concentration difference may explain why pyridoxal phosphate failed to elicit a selective pattern of EOG inhibition in the frog, even though such behaviour had been previously observed in the rat.

3 α-Methyl mannoside binds to the saccharide site in concanavalin A; it can inhibit, and sometimes reverse, the binding of Con A to receptors which involve the sugar-binding site of the lectin (Goldstein and Hayes, 1978).
to the normal superfusion medium" (Shirley et al., 1986). This implies that the interaction of Con A with the sensory membranes involves the sugar-binding site of a glyco-molecule (presumably a protein) (see Bittiger and Schnebli, 1976). Recently, four glyco-proteins, which may be "involved in vertebrate olfactory reception" have been identified amongst seven olfactory-specific polypeptides extracted from isolated frog cilia (Chen and Lancet, 1984; also see Lancet and Chen, 1984).

It has been previously suggested that odorants may act as allosteric ligands and that multiple receptor molecules may exist for a particular odorant (e.g. see Polak, 1973; Dodd and Persaud, 1981). The inability of concanavalin A, even at high concentrations, to completely abolish the EOG signals obtained to sweaty-smelling fatty acids in either the frog or the rat (Shirley et al., 1986) can be regarded as evidence for multiple receptors directed against this class of odorants. Multiple receptors, which have now been found for several types of pharmacologically active molecules, such as dopamine (Kebabian and Calne, 1979) and serotonin (Peroutka and Snyder, 1983), may be a general feature of vertebrate chemoreceptors. The vapour-phase labelling studies previously described (see Chapter 4) have suggested the existence of multiple olfactory receptors for the heterocyclic odorants examined.

The carboxylic odorants, unlike most other odorant classes, possess a polar group with a low pK<sub>a</sub>. Consequently, under physiological conditions, the acids will be predominantly ionised in the mucus. It may therefore be speculated that a receptor site exists in the olfactory cells which can recognise the carboxylate anion.

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1 The olfactory-specific polypeptides amounted to at least 70% of the total ciliary membrane protein.
Furthermore, since the distinctive "sweaty-foot" odour is characteristic of the C₄ - C₅ alkanolic acids, it may also be suggested that the carboxylate receptor contains a sub-site which recognises the appropriately shaped alkyl group. The results obtained from both the frog and the rat (Shirley et al, 1983b, 1986) are compatible with such ideas, although not unequivocally so.

The present studies with concanavalin A have served to highlight the differences that exist between the in vivo frog and in vitro rat preparations (see Appendix 2). In particular, it is worthy of note that the BOG peak amplitudes obtained from the rat were generally larger than those recorded from the frog. Further, the in vitro preparations exhibited stable BOG responses more quickly than the in vivo preparations, both before and after treatment with the modifying reagent. However, the frog preparations tended to possess greater longevity than their in vitro counterparts.

A typical experiment on the rat could be completed within 1 - 2 hours, whilst the in vivo studies routinely extended over 3 - 4 hours.
CHAPTER 6: LIGAND BINDING STUDIES

6.1 Introduction

6.1(1) Theoretical aspects of ligand binding

The binding of a ligand to a receptor possessing N binding sites can be described by the equilibria:

\[ R + X \rightleftharpoons K_1 RX \]  
\[ RX_1 + X \rightleftharpoons K_1 RX_1 \]  
\[ RX_{N-1} + X \rightleftharpoons K_N RX_N \]

where \( R \) is the receptor, \( X \) is the ligand, and \( K_1, \ldots, K_N \) represent the appropriate thermodynamic dissociation constants. The dissociation constant, of which the reciprocal is the association constant, provides information on the concentration of reactants necessary to form half maximal amounts of complex (Levitzki, 1980).

When only a single class of receptor site is present in the receptor population (i.e. no heterogeneity is found and no co-operativity amongst the receptor sites is observed), the average number of ligand molecules bound to the receptor molecule is given by:

\[ N_x = \frac{N}{K_D + [X]} \]  

where \( K_D \) is the ligand-receptor dissociation constant.

Re-arrangement of equation (4) yields:

\[ \frac{N_x}{[X]} = \frac{N}{K_D} - \frac{N_x}{K_D} \]  

Various theoretical and practical aspects of receptor binding have been discussed elsewhere (e.g. see Quatreshas and Hollenberg, 1976; Levitzki, 1980; Ferguson-Miller and Koppenol, 1981; Titerle, 1981; Hollenberg and Nexé, 1981); for a comprehensive and systematic account of receptor theory, see Boeynaems and Dumont (1980).
The plot of $N_{X}/[X]$ versus $N_{X}$ is known as the Scatchard plot (Scatchard, 1949) and is one of the most sensitive graphical methods available for the presentation of ligand binding results, since it clearly demonstrates the precision of the data.

6.1(ii) Assay of receptors

It is generally acknowledged that numerous ligands can interact in a specific manner with naturally occurring non-receptor macromolecules (Goldstein et al, 1974); such interactions often exhibit both a high affinity and some degree of chemical or steric specificity. Thus, in order to identify a true ligand-receptor interaction, several criteria must be met (Table 6.1) (Cuatrecasas and Hollenberg, 1976; Titeler, 1981).

Receptor binding studies usually employ compounds (i.e. ligands) of high specific radioactivity that bind to the putative receptors with measurable affinity. Following equilibration of the receptor tissue preparation with the radiolabelled ligand, it is necessary to separate the unbound compound from the bound ligand. The speed with which this separation must be achieved depends upon the lifetime of the ligand-receptor complex (Cuatrecasas and Hollenberg, 1976). In general, centrifugation and filtration techniques have proved most useful for the rapid, efficient separation of membrane-bound from free ligand. Other techniques, including equilibrium dialysis and equilibrium gel filtration (Hummel and Dreyer, 1962; Cuatrecasas et al, 1967) have also been employed.

It is necessary to differentiate between specific and non-specific binding. In most cases (except for intracellular steroid and thyroid hormone receptors), one measures the binding of a ligand to an impure receptor embedded in a membrane matrix. However, the ligand itself may bind to various components of the receptor tissue, as well
Table 6.1 Criteria for receptor identification

It is desirable to examine:

1. the binding of analogues and/or antagonists

2. saturability and affinity of binding

3. the reversibility of binding

4. the tissue distribution of specific binding

5. the simultaneous correlation of the binding data with the biological dose-response curves in identical tissue preparations.

(Cuatrecasas and Hollenberg, 1976)
as to filters, glassware and other non-receptor materials; this binding, which is normally of low affinity and is non-saturable, is known as "non-specific" binding. The number of non-specific sites is usually very large compared to the number of specific binding sites. Since non-specific interactions are normally characterised by low affinity processes, the amount of radioactive ligand binding to such sites tends to increase linearly with increasing concentrations of free ligand (Figure 6.1).

Estimations of the extent of non-specific binding may be obtained by measuring the binding of the radioactive ligand under conditions where it cannot bind to the specific receptor sites. Such conditions are met when the binding of the radioactive ligand is measured in the presence of a large excess concentration (ten-fold or more the dissociation constant) of the non-radioactive ligand, or in the presence of high concentrations of another competitive ligand. Thus, the binding isotherm to the specific receptor sites is obtained by subtracting the non-specific binding from the total amount bound (Figure 6.1) (Levitzki, 1980; Titeler, 1981). It follows, therefore, that the amount of non-specific binding determines the precision with which the specific binding isotherm may be obtained (see Figure 6.2).

6.1(iii) Odorant binding approach to receptor identification

The experimental difficulties encountered in studies of hormone receptors and neurotransmitters (Cuatrecasas and Hollenberg, 1976) are

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1 E.g. non-receptor ("non-specific") interactions which shared, at least superficially, the criteria (saturability, specificity, high affinity and reversibility) commonly attributed to "specific" hormone-receptor interactions, have been reported for the binding of 125I-insulin to various non-receptor materials (talc, silica, protein-agarose derivitaves, glass test-tubes) (Cuatrecasas and Hollenberg, 1975).

2 For exceptions, see Cuatrecasas and Hollenberg (1976).
Figure 6.1  Typical appearance of ligand binding data

Graphical presentation of the actual binding data of $^{125}$I hydroxybenzylpindolol ($^{125}$I-HYP) binding to turkey erythrocyte $\beta$-adrenergic receptors.

(a) $\bullet$- - - total binding; o-o-o, binding in the presence of $5.0 \times 10^{-6}$ dl-propranolol representing the non-specific binding.

(b) The specific binding obtained by subtracting the non-specific binding (o-o-o) from the total binding (---) in (a)

Taken from Levitzki (1980)
Aliquots of homogenates of hearts were incubated with various concentrations of $[^3H]$ QNB at room temperature for 60 min. prior to the separation of bound from free ligand by ultrafiltration. Saturating concentrations of oxotremorine were employed to monitor non-specific binding.

- o: 9-day-old hearts
- *: 3-day-old hearts
- Δ: 9-day-old hearts plus 100 μM oxotremorine

Taken from Galper et al (1977)
fewer than those found with the vertebrate olfactory system. A major problem concerns the nature of ligands that are effective as odorants (see Section 1.4(i)). When dealing with air-breathing vertebrates, the predominating structural requirement for such ligands is that they be sufficiently volatile to reach the olfactory epithelium\(^1\) (Dodd and Persaud, 1981). Consequently, odorants tend to be low molecular weight compounds of a hydrophobic/amphiphilic nature, which thus exhibit considerable amounts of non-specific binding to membranes and proteins. This makes the identification of receptors through binding studies very difficult in homogenates and membrane fractions. However, the reversible labelling of olfactory receptors in the intact tissue, followed by tissue fractionation, may prove a suitable procedure for their isolation and purification; such an approach has been successfully followed in the study of acetylcholine receptors (Eldefrawi and Eldefrawi, 1977). Although the interaction of water-soluble odorants (i.e. some \(\alpha\)-amino acids) with fish olfactory membranes exhibits little non-specific binding, thereby permitting the identification of olfactory receptors (Cagan and Zeiger, 1978; but also see Brown and Hara, 1981), such identification generally requires information from several types of experiments other than ligand binding studies. The ability to recognise a drug or hormone receptor by correlating the \textit{in vitro} binding affinities of agonists or antagonists with the biological activity of the system \textit{in vivo} renders the isolation of such receptors easier than that of receptor molecules from the olfactory system (Dodd and Persaud, 1981). The experimental strategies applicable to

\(^1\) This is not applicable to fish (e.g. rainbow trout), which can perceive as odorants many water-soluble amino acids (see Cagan and Zeiger, 1978; Rhein and Cagan, 1980, 1981; Cagan and Rhein, 1980; also see Arzt et al, 1984).
the isolation and study of olfactory receptors have recently been reviewed (Dodd and Persaud, 1981; see Table 6.2).

Several attempts have been made to isolate pure plasma membrane fragments of the olfactory neurones. Koch (1969, 1973) used differential- and sucrose-density-gradient centrifugation techniques to separate subcellular components from rabbit brain and olfactory tissue homogenates; membranes from various cell fractions were identified by the enzyme markers, cytochrome oxidase and Na\(^+\)-K\(^+\)-ATPase. However, subsequent investigations using the same techniques failed to achieve clear separations of neuronal plasma membranes (see Koyama et al, 1971; Menco et al, 1974; Menevse, 1977; Menco, 1977a). In the absence of such preparations, studies employing homogenates of olfactory epithelium have sought to identify components (i.e. proteins) which interact with odorants (Table 6.3; also see Novoselov et al, 1980).

Investigations into the binding of several odorant amino acids to fish olfactory membranes have led to the postulate that "a multiplicity of types of olfactory binding sites exist in the trout" (Cagan and Zeiger, 1978). Support has also been provided for the hypothesis that odorant recognition sites are integral parts of the cilia (Cagan and Rhein, 1980; Rhein and Cagan, 1980, 1981; also see Section 1.3(i)). Recently Novoselov et al (1980) have identified structures (molecular weight about 120,000) in the membrane fraction of skate olfactory tissue which exhibit a high affinity for alanine\(^1\).

The membrane fraction of rat and frog olfactory tissue scrapings has been reported to contain acceptor sites capable of

\(^1\) The value of the measured dissociation constant (\(K_D = 0.13 \times 10^{-9}\)M) was "considerably lower" than that reported for this amino acid receptor from trout (\(K_D = 5.6 \times 10^{-6}\)M; see Cagan and Zeiger, 1978).
### Table 6.2 Experimental strategies applicable to biochemical studies of olfaction

<table>
<thead>
<tr>
<th>Level</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact epithelium</td>
<td>Experiments can be monitored electrophysiologically</td>
</tr>
<tr>
<td>Isolated “intact” neurons</td>
<td>If electrophysiological methods are not applicable, spectroscopic methods should be used to monitor the experiments</td>
</tr>
<tr>
<td>Homogenates from epithelium</td>
<td>Electrophysiological methods not applicable at this level</td>
</tr>
<tr>
<td>Sensory membranes</td>
<td>The potential across membrane vesicles can be measured by spectroscopic probes</td>
</tr>
<tr>
<td>Receptor proteins</td>
<td>Can be “reconstituted” into membranes</td>
</tr>
<tr>
<td>Receptor phospholipids</td>
<td>Membrane vesicles can be used to study properties of the lipids</td>
</tr>
</tbody>
</table>

Two key biochemical experiments - (1) odorant binding to determine the number of specific binding sites, and (2) specific labelling of olfactory receptors - can be carried out at the levels suggested in the table.

* Taken from Dodd and Persaud (1981) *
### Table 6.3  Odorant binding studies performed on the vertebrate olfactory system

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Tissue</th>
<th>Binding parameters</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 radio-actively labelled odorant amino acids</td>
<td>a sedimentable fraction derived from the olfactory rosettes of the rainbow trout, <em>Salmo gairdneri</em></td>
<td>Scatchard analyses gave evidence of two types of binding sites for most of the amino acids studied: $K_D = 10^{-9}M$, $K_D = 10^{-5}M$</td>
<td>Cagan and Zeiger (1978); Also see Cagan and Rhein (1980); Rhein and Cagan (1980, 1981)</td>
</tr>
<tr>
<td>$[^3H]_L$-camphor</td>
<td>the membrane fraction of rat and frog olfactory tissue scrapings</td>
<td>$K_D = 1.5 \times 10^{-9}M$</td>
<td>Fesenko et al (1978, 1979)</td>
</tr>
<tr>
<td>$[^3H]_L$-ala(\text{(n)}}</td>
<td>the membrane fraction of skate (<em>Dasyatis pastinaca</em>) olfactory epithelium tissue homogenate</td>
<td>$K_D = 0.13 \times 10^{-9}M$, $n_1 = 0.18$ pmol per mg of protein, $K_D = 3 \times 10^{-9}M$, $n_2 = 15$ pmol per mg of protein</td>
<td>Novoselov et al (1980)</td>
</tr>
<tr>
<td>$[^3H]_50\text{(\alpha)}$-androst-16-en-3-one</td>
<td>sow olfactory tissue homogenate</td>
<td>$K_D = 1.2 \times 10^{-9}M$, $n = 3.3$ pmol per mg of protein</td>
<td>Gennings et al (1977)</td>
</tr>
<tr>
<td>$[^3H]_50\text{(\alpha)}$-androstan-3-one</td>
<td>rabbit and cow olfactory mucosa homogenate, sow olfactory tissue homogenate, sheep olfactory tissue homogenate</td>
<td>$K_D = 60 \times 10^{-9}M$, $n = 32$ pmol per mg of protein, $K_D = 40 \times 10^{-9}M$, $n = 28$ pmol per mg of protein, $K_D = 1.43 \times 10^{-9}M$</td>
<td>Persaud (1977), Persaud (1980), Persaud et al (1980)</td>
</tr>
<tr>
<td>$[^3H]_2\text{(\alpha)}$-isobutyl-3-methoxy-pyrazine</td>
<td>cow olfactory mucosa homogenate</td>
<td>$K_D = 1.2 \times 10^{-6}M$, $n = 270$ pmol per mg of protein</td>
<td>Felosi et al (1980, 1981, 1982)</td>
</tr>
</tbody>
</table>

1 Also see Hancock, M.R., Gennings, J.N. and Gower, D.B. (1985) 'On the existence of receptors to the pheromonal steroid, 5α-androst-16-en-3-one, in porcine nasal epithelium' FEBS Lett. 181, 328-334
binding camphor with a high affinity \((K_D = 1.5 \times 10^{-9} \text{M})\); Fesenko et al., 1978). These structures, which were absent in other tissues studied (e.g. tongue, lung, brain and liver), were suggested as belonging to a class of receptor molecules for camphoraceous odorants. Camphor-binding activity in the rat olfactory epithelium was abolished by pronase and trypsin (by 65% and 40% respectively) and was reduced by sulphydryl reagents (by 5-8-fold); maximum binding was observed at pH 7.4 (Fesenko et al., 1979). These results indicated that the binding component (molecular weight about 120,000) was proteinaceous in nature. However, as with other binding studies, no evidence has yet been presented to confirm that this binding component is indeed an olfactory receptor (Dodd and Persaud, 1961).

Investigations into the binding of the boar pheromone, 5α-androst-16-en-3-one to sow olfactory tissue homogenates have provided tentative evidence for a receptor which exhibits saturable specific binding of this odorant \((K_A = 8.3 \times 10^8 \text{M})\) (Gennings et al., 1977; also see Section 1.3(ii)c). However, subsequent studies (Persaud, 1977, 1980; Pelosi et al., 1978; Persaud et al., 1980) have failed to reproduce these findings.

The binding of the urinous odorant 5α-androstan-3-one to homogenates of sheep olfactory epithelium has been investigated by Persaud (1980) (also see Persaud et al., 1980). Preliminary work showed that this compound, which is closely related to 5α-androst-16-en-3-one (Gennings et al., 1977), could be perceived as an odorant by sheep; despite its extreme hydrophobicity and relative non-volatility, small EGG responses were obtained to vapour-phase concentrations of this odorant from an in vitro preparation of sheep olfactory epithelium (Figure 6.3(A); Squirrell, 1978; see Section 6.4(iii)). Binding studies utilising crude olfactory homogenates proved
Figure 6.3 Binding of 5α-[16,17-3H]androstane-3-one to sheep olfactory epithelium

(A) EOG from intact sheep olfactory epithelium in response to stimulation by androstanone and amyl acetate.

(B) Binding of androstanone to the supernatant fractions of sheep olfactory and respiratory epithelia, following centrifugation at 13,000 x g for 15 min. The non-specific binding (0,0) was determined by adding excess unlabelled androstanone to the tissue and labelled steroid. The free and bound ligands were separated using the standard charcoal method.

(C) Scatchard plot of binding data shown in Figure 6.3B.

Taken from Dodd and Persaud (1981)
inconclusive and irreproducible, due mainly to the extreme hydrophobicity of the ligand. However, investigations employing a 13,000 x g supernatant fraction of olfactory tissue homogenate demonstrated specific, saturable binding ($K_A = 7.0 \times 10^8$ M; Figures 6.3(B) and 6.3(C)). This binding was inhibited by several ligands (Table 6.4). It should be noted that all these studies were complicated by the presence of large amounts of non-specific binding which considerably decreased the sensitivity and accuracy of the assay. Specific binding was not observed with tissues such as respiratory epithelium, brain and liver.

As yet, it is not clear whether the observed binding reflects the presence of a specific olfactory receptor protein, or a steroid hormone-binding protein.

In an attempt to facilitate the isolation of olfactory cells, Hirsch and Margolis (1981) have investigated the specific binding of a number of radioligands to homogenates of mouse olfactory epithelium; it was reasoned that if certain ligands could be shown to bind to olfactory neurones, then such ligands could be coupled to a solid support (e.g. Sepharose) to yield an affinity column (see Price, 1978, 1981). The ligands employed were drugs known to bind to various neurotransmitter and drug receptors in the olfactory bulb and brain (Hirsch et al., 1978; Hirsch and Margolis, 1979; Nadi et al., 1980; also see Blaha et al., 1984).

The mouse olfactory epithelium was found to contain binding sites for $[^3H]$ diazepam (a benzodiazepine; also see Anholt et al., 1984); $[^3H]$ clonidine, WB-4101, dihydroergocryptine ($\alpha$-adrenergic ligands); $[^3H]$ dihydroalprenolol (a $\beta$-adrenergic ligand);

1 $2'-(2',6'$-dimethoxy)phenoxyethylamino)methyl benzodioxan
Table 6.4

Specificity of binding of 5a-androstan-3-one to homogenates of sheep olfactory epithelium

5α-Androstan-3-one binding is inhibited by the following ligands in the rank order shown:

1. 5α-androst-16-en-3-one (urinous)

2. cis-2-methyl-2-(4′-tert-butylcyclohexyl)pentan-4-one (urinous)

3. 5α-androst-16-en-3α-ol (sandalwood/musk)

4. 5α-androst-16-en-3β-ol (nearly odourless)

(Dodd and Persaud, 1981)
[\textsuperscript{3}H]quinuclidinyl benzilate ([\textsuperscript{3}H]QNB; a muscarinic cholinergic ligand) and L-[\textsuperscript{3}H] carnosine (the olfactory peptide neurotransmitter candidate, see Section 7.1(ii))\textsuperscript{1}. Except for the [\textsuperscript{3}H] diazepam site, which was the "non brain" type (Braestrup and Squires, 1977), and the L-[\textsuperscript{3}H] carnosine site, which was not saturable, the binding sites possessed pharmacological characteristics similar to sites in the brain (Snyder and Bennett, 1976). The epithelium was noted to be devoid of specific opiate binding ([\textsuperscript{3}H] dihydromorphine), GABA binding ([\textsuperscript{3}H] muscimol) and kainic acid binding ([\textsuperscript{3}H] kainic acid). Additional studies employing mice which had been subjected to unilateral olfactory bulbectomies suggested that the [\textsuperscript{3}H] quinuclidinyl benzilate binding sites (muscarinic cholinergic binding sites) were associated with the olfactory neurones (Hirsch and Margolis, 1981; see Table 6.5).

As an extension of previous work on muscarinic receptor mechanisms in other tissues (e.g. see Hedlund and Bartfai, 1979; Lundberg et al., 1982; Hedlund et al., 1983), the muscarinic receptors in the salamander olfactory epithelium have recently been characterised: they were found to specifically bind [\textsuperscript{3}H]QNB with an affinity of 0.75 nM (0.3 pmol [\textsuperscript{3}H]QNB/mg protein) (Hedlund and Shepherd, 1983; Hedlund et al., 1984). Investigations into the ability of four odorants (amyl acetate, butanol, camphor and dimethylsulphide; see Getchell and Shepherd, 1978; Fesenko et al., 1979) to interact with [\textsuperscript{3}H]QNB binding revealed a close correlation between muscarinic

\textsuperscript{1} Derby et al. (1984) have recently presented electrophysiological and behavioural evidence for the existence of purinergic receptor cells in the olfactory systems of two marine invertebrates.
Table 6.5  Effect of unilateral olfactory bulbectomy on $^3$H-labelled ligand binding in mouse olfactory epithelia

<table>
<thead>
<tr>
<th>$^3$H-labeled ligand (nM)</th>
<th>Exp. No.</th>
<th>Normal side</th>
<th>Unoperated side</th>
<th>Bulbectomized side</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^3]$H]Quinuclidinyl benzilate (1.0)</td>
<td>1</td>
<td>54</td>
<td>50</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>70</td>
<td>73</td>
<td>27</td>
</tr>
<tr>
<td>$[^3]$H]Clonidine (3.7)</td>
<td>1</td>
<td>95</td>
<td>128</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>52</td>
<td>65</td>
<td>98</td>
</tr>
<tr>
<td>$[^3]$H]Dihydroergocryptine (5.0)</td>
<td>1</td>
<td>46</td>
<td>118</td>
<td>188</td>
</tr>
</tbody>
</table>

Unilateral olfactory bulbectomies were performed 30 days before the binding assays were performed. Normal refers to unoperated control animals. Unoperated side refers to the portion of epithelium obtained contralaterally to the removed bulb. Bulbectomized side refers to the portion of epithelium obtained ipsilaterally to the removed bulb. Specific binding of $[^3]$H quinuclidinyl benzilate was determined with and without 1/AM atropine, and specific binding of $[^3]$H clonidine and $[^3]$H dihydroergocryptine were determined with and without 10/AM phentolamine. Results are means of triplicate determinations which varied about 15%.

Taken from Hirsch and Bargolis (1981)
receptor and olfactory receptor binding\(^1\); at 0.01 mM and 0.1 mM concentrations\(^2\), the odorants were found to inhibit \[^3H\]QNB binding such that a shift in this binding was induced from an apparent recognition of one receptor binding site to the recognition of two receptor sites with different affinities and capacities. No effect on \[^3H\]QNB binding was observed with two non-odorants (quinoline and sucrose) nor with odorants employed in control experiments performed on an homogenate of the salamander brain. Additional studies have suggested that \[^3H\]QNB and \[^3H\]amyl acetate interact by binding either to two closely associated membrane receptors or to different subunits of the same salamander epithelial receptor, and that "these questions point the way to more extensive studies including purification of olfactory receptors" (Hedlund et al, 1984).

The potent bell-pepper odorant 2-isobutyl-3-methoxypyrazine has recently been reported to bind to cow olfactory mucosa homogenate (Pelosi et al, 1980, 1981, 1982). This binding, which was saturable in the micromolar range (\(K_D = 1.2 \times 10^{-6}\) M), was shown to be competitively inhibited by other bell-pepper odorants, but not by pyrazines that possessed different odours (Pelosi et al, 1980, 1982). The disappearance of binding activity after treatment of the mucosa homogenate with proteolytic enzymes or with a SDS-containing buffer, indicated that the binding component was proteinaceous in nature.

\(^1\) This may indicate a modulatory role for acetylcholine, acting on muscarinic receptors, in the primary olfactory response. Consideration "should also be directed to a possible role of acetylcholine and muscarinic receptors in the control of receptor cell differentiation from basal stem cells (Sastry and Sadavongvivad, 1979) and control of movement of the olfactory cilia, in analogy with other systems (Sastry and Sadavongvivad, 1979; Slaughter and Aiello, 1982)" (Hedlund et al, 1984).

\(^2\) Odorant concentrations of 0.01 mM and 0.1 mM are within the range previously employed in physiological experiments (Getchell and Shepherd, 1978).
(Pelosi et al, 1981). Other tissues did not bind 2-isobutyl-3-methoxypyrazine to a "significant extent" (Pelosi et al, 1982).

This chapter describes the binding of $[^{3}H]2$-isobutyl-3-methoxypyrazine to a 13,000 x g supernatant fraction of sheep olfactory tissue homogenate.
6.2 The ligand: $[^3]$H-2-isobutyl-3-methoxypyrazine (Figure 6.4)

6.2.1 Introduction

2-Isobutyl-3-methoxypyrazine appears particularly well suited to ligand-binding investigations; the extremely low olfactory threshold (0.002 parts/10$^9$; Teranishi et al., 1974) suggests a probable high affinity for the appropriate receptor sites. Moreover, the olfactory data available for other compounds of similar structures (e.g. see Buttery et al., 1969; Seifert et al., 1970, 1972; Maga and Sizer, 1973, 1975; Parliment and Epstein, 1973; Teranishi et al., 1974; Takken et al., 1975; Calabretta, 1978) provide a useful basis for biochemical studies on the specificity of such sites (Pelosi et al., 1982). A specific anosmia$^1$ to 2-isobutyl-3-methoxypyrazine has been tentatively identified (P. Pelosi, personal communication) and so this pyrazine may be regarded as a "primary odour" which interacts with specific receptor sites$^2$ (e.g. see Amoore, 1977, also see Section 1.3(ii)c).

2-Isobutyl-3-methoxypyrazine possesses a characteristic green bell pepper odour of great intensity and pervasiveness$^3$ (Buttery et al., 1969; Seifert et al., 1970; Teranishi et al., 1974).

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$^1$ It should be noted that, at present, little direct correlation is possible between the values obtained in vitro for ligand binding constants and the in vivo responses observed to various odorants: in vitro measurements are made on the olfactory mucosa excised from an animal, whilst in vivo behavioural studies, such as threshold determinations, odour descriptions and studies of specific anosmias, have been performed mainly with humans (Pelosi et al., 1981).

$^2$ The primary odorants which have been tentatively identified (Amoore, 1971, 1974) may be compounds that interact with only a very few types of receptor sites, with one type predominating (Getchell and Getchell, 1974).

$^3$ For a brief discussion of pyrazine odorants see Section 1.4(ii).
2-isobutyl-3-methoxypyrazine possesses:

(1) Characteristic green bell pepper odour

(2) Extremely low olfactory threshold

\(0.002\) parts/\(10^9\)

(Teranishi et al, 1974)
Experimental methodology

Preparation and characterisation of $[^3\text{H}]2$-isobutyl-3-methoxypyrazine

A sample of $[^3\text{H}]2$-isobutyl-3-methoxypyrazine ($[^3\text{H}]$pyrazine) (10 M in 1,2-propanediol; total volume about 5 ml) was generously donated by Dr. Paolo Felosi, University of Pisa, Italy; its preparation and subsequent purification have been previously described (see Pelosi et al., 1981). Aliquots of this "stock solution" were diluted with buffer (10 mM HEPES buffer, pH 7.4, containing EDTA (1 mM) and bovine serum albumin (5 mg/ml)) as required to form the "working solution" (concentration of $[^3\text{H}]$pyrazine = $10^{-6}$M; see Section 6.3(iv)).

The purity (chemical and radiochemical) of the tritiated pyrazine was checked and the specific radioactivity determined (2.08 Ci/mole; P. Pelosi, personal communication; Pelosi et al., 1981, 1982) prior to donation (see Pelosi et al., 1981; also see Appendix 3). These characteristics (not including radiochemical purity; see Section 6.2(ii)b) were also checked periodically throughout the course of the present study.

Purity of $[^3\text{H}]2$-isobutyl-3-methoxypyrazine

The methodology used to check the purity of the $[^3\text{H}]2$-isobutyl-3-methoxypyrazine was similar to that employed by Persaud (1980) in the determination of $[^3\text{H}]16,17$-androstan-3-one purity.

A plastic-backed silica gel (0.2 mm thick; pre-coated) thin layer chromatography (t.l.c.) plate (5 cm x 10 cm) was longitudinally bisected and divided up into 1 cm squares. Non-radioactive 2-isobutyl-3-methoxypyrazine (1 µl) was spotted onto one half of the plate, whilst $[^3\text{H}]$pyrazine (approximately 0.5 µl; $10^{-6}$M in 1,2-propanediol) together with unlabelled compound ($1$) (approximately 0.5 µl) were spotted onto the

1 The non-radioactive 2-isobutyl-3-methoxypyrazine acted as a carrier for the tritiated derivative, which was dissolved in 1,2-propanediol.
other half (Figure 6.5).

The t.l.c. plate was developed by ascending chromatography in toluene/chloroform/acetone (2 : 2 : 1; total volume = 10 ml), after which it was cut along the line of bisection. The non-radioactive side of the plate was sprayed with Allen's reagent\(^1\) and placed in an oven at 110\(^\circ\)C for about 10 minutes; the 2-isobutyl-3-methoxypyrazine was visualised as a brown spot. Confirmatory detection was achieved by ultraviolet illumination of the plate at 254\(\mu\). The \(R_f\) value for the substituted pyrazine was found to be about 0.55 (Figure 6.5).

The radioactive side of the t.l.c. plate was cut up into 1 cm squares, and each square was placed in a plastic scintillation mini-vial containing Bray's fluid\(^2\) (4 ml) (Bray, 1960). In addition, scintillation vials containing squares of unused t.l.c. plate were prepared in order to provide a baseline control for any background luminescence (Peng, 1977). After cooling in the dark, the mini-vials were counted in a Packard Prias liquid scintillation spectrometer, with a counting efficiency for tritium of about 53\%. The counting profile located a peak of radioactivity at a position corresponding to the previously determined non-radioactive 2-isobutyl-3-methoxypyrazine. No other appreciable amounts of radioactivity were detected (Figure 6.6).

The chromatographic solvent system was validated by its ability to demonstrate similar \(R_f\) values for 2-isobutyl-3-methoxypyrazine when this compound was applied to the t.l.c. plate under a variety of conditions (see Figure 6.7).

Although these observations served to verify the nature of the \(\[^3\text{H}\]pyrazine, its radiochemical purity should ideally have been


\(^2\) Butyl-FBD (7g), Triton X - 100 (500 ml) and toluene (1 litre) (Bray, 1960).
Figure 6.5 Determination of the purity of $[^3H]2$-isobutyl-3-methoxypyrazine

The loaded t.l.c. plate was developed in toluene/chloroform/acetone (2:2:1) and then cut longitudinally along the line of bisection. 2-Isobutyl-3-methoxypyrazine was visualised with Allen's reagent and by ultraviolet illumination at 254 nm ($R_F = 0.55$) whilst $[^3H]2$-isobutyl-3-methoxypyrazine was located using a liquid scintillation spectrometer ($R_F = 0.55$).
After leading with $^{3H}$2-isobutyl-3-methoxypyrazine (together with unlabelled carrier) and developing in toluene/chloroform/acetone (2:2:1), the t.l.c. plate was cut up into 1 cm squares and analysed in a liquid scintillation spectrometer. The position of the radioactive peak ($R_F = 0.55$) is equivalent to that determined (see Figure 6.5) for 2-isobutyl-3-methoxypyrazine ($R_F = 0.55$).
Figure 6.7  Thin-layer chromatography of 2-isobutyl-3-methoxypyrazine and trimethylpyrazine

The t.l.c. plates were loaded with:

(a) 2-isobutyl-3-methoxypyrazine (1μl)
(b) 2-isobutyl-3-methoxypyrazine in 1,2-propanediol (1 : 11; 1μl)
(c) trimethylpyrazine and 2-isobutyl-3-methoxypyrazine in 1,2-propanediol (1 : 1 : 10; 1μl)

Development occurred in toluene/chloroform/acetone (2 : 2 : 1)

The pyrazine compounds were visualised with Allen's reagent and by ultraviolet illumination at 254μ.
cross-checked by an independent technique such as radio-GLC or radio-HPLC. Unfortunately, however, these facilities were not available in the present study. Consequently, confirmation of the purity of the $[^3H]2$-isobutyl-3-methoxypyrazine rested upon the finding that chromatography in three different solvent systems gave identical results (see Persaud, 1980).

6.2(ii) Specific radioactivity of $[^3H]2$-isobutyl-3-methoxypyrazine

The donated "stock solution" of $[^3H]2$-isobutyl-3-methoxypyrazine ($10^{-4}M$ in 1,2-propanediol; P. Pelosi, personal communication) was diluted with buffer (10 mM HEPES buffer, pH 7.4, containing EDTA (1 mM) and bovine serum albumin (5 mg/ml)) to form the "working solution" (concentration of $[^3H]pyrazine = 10^{-6}M$) (see Section 6.3(iv)), various aliquots of which were then dispensed into a set of plastic scintillation mini-vials containing Bray's fluid (4 ml per vial) (Bray, 1960). After cooling in the dark, the vials were each counted for 10 minutes in a Packard Trias liquid scintillation spectrometer.

The $[^3H]2$-isobutyl-3-methoxypyrazine was found to possess a specific radioactivity of 2.05 Ci/mole (Figure 6.8), which is in good agreement with the previously reported value of 2.08 Ci/mole (Pelosi et al, 1981, 1982). However, it should be noted that the accuracy of this estimate depends upon the precision of the independently-determined value for the concentration of $[^3H]pyrazine in the "stock solution" ($10^{-4}M$; see Appendix 3). As noted previously (see

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1 The presence of both chemical and radiochemical impurities in different lots of $[1,2-^3H]$cholesterol and $[24,25-^3H]$cholesterol which had been previously found to be radiochemically pure by conventional chemical and other in vitro tests, has recently been reported (Davison et al, 1980).

2 Toluene/chloroform/acetone (2 : 2 : 1)
Toluene/diethyl ether (3 : 2)
Cyclohexane/ethanol (1 : 1)
Aliquots of $[^3\text{H}]\text{2-isobutyl-3-methoxypyrazine}$ ($10^{-6}\text{M}$) in 10 mM HEPES buffer, pH 7.4, containing EDTA (1 mM) and bovine serum albumin (5 mg/ml) were dispensed into plastic scintillation mini-vials containing Bray's fluid and counted in a liquid scintillation spectrometer. Each point represents the mean d.p.m. value of triplicate mini-vials.
Section 6.2(ii)b), it was not feasible to cross-check this value in the present study. Consequently, there is a possibility that the accompanying results may carry a systematic error.
6.3 **Methods**

6.3(i) **Tissue preparation**

Sheep heads were obtained from a local abattoir within 5 minutes of death; their dissection and subsequent tissue fractionation took about 2 hours.

The severed head was cut longitudinally to one side of the midline, and the nasal septum was removed to expose the olfactory mucosa which was readily identifiable by its yellowish-brown pigmentation (see Section 1.1(i)). This was stripped away from the turbinates and suspended (1 g wet weight/10 ml buffer) in cold 20 mM HEPES buffer, pH 7.4, containing EDTA (1 mM), at 4°C, using a glass Potter-Elvehjem homogeniser (0.13 - 0.18 mm clearance) with a motorised teflon pestle (7 x 3 strokes with intermittent cooling on ice). Next, the suspension was centrifuged at 600 x g for 5 minutes at 4°C in a Sorvall RC2B refrigerated centrifuge to remove cell debris and connective tissue. The resulting supernatant was subjected to further centrifugation at 13,000 x g for 20 minutes at 4°C (see Koch, 1969). Most of the binding studies described in this chapter were performed on the supernatant (microsomal) fraction obtained from this centrifugation procedure. The supernatant was stored in 1 ml aliquots at -20°C, under which conditions it remained viable for several weeks.

Normally, the olfactory mucosa from at least three animals was pooled prior to homogenisation and centrifugation. Any sheep possessing rhinitic infections of the olfactory tissue were discarded.

For some experiments, portions of the brain and the respiratory

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1 For a comparison of preparation viability, some tissue was periodically suspended in 0.32 M sucrose containing EDTA (1 mM), as described by Koch (1969). However, no differences were detected in the binding characteristics exhibited by the alternatively prepared tissue supernatant fractions.
epithelium were also removed and fractionated as described; liver obtained from sheep was similarly treated.

6.3(i) Gel chromatography

All experiments were performed at 4°C. Columns of Sephacryl S200 Superfine (0.9 x 30.0 cm), equilibrated with 10 mM HEPES buffer, pH 7.4, containing EDTA (1 mM), were calibrated for molecular weight determination with standard proteins (Andrews, 1961). Blue Dextran 2000 (2 mg/ml) was used to check the homogeneity of the packed beds and to determine the columnar void volumes. Column eluates were scanned for ultraviolet absorption (L.K.B. Uvicord Type 470A control unit) and were collected in fractions (Gilson Microcol TDC 80 fraction collector).

6.3(ii) Protein estimation

Protein was estimated by the method of Lowry et al (1951).

6.3(iv) Ligand binding assays

The assays were performed in Pyrex test-tubes (10 mm x 75 mm; see Section 6.4(i)a) in the presence of 10 mM HEPES buffer, pH 7.4, containing EDTA (1 mM) and bovine serum albumin (5 mg/ml).

A working solution of \([3H]2\)-isobutyl-3-methoxypyrazine was prepared by dispensing an aliquot of the stock solution (10⁻⁴M in 1,2-propanediol) into a suitable volume of the assay buffer. Portions of this working solution were then introduced to buffer contained in the assay tubes such that the total volume of liquid in each tube was 100µl. After cooling on ice, a sample (50µl; usually 0.1 mg) of the tissue preparation was added to each tube, which in turn was

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1 HEPES (pKₐ 7.55) was employed because of its exceptional buffering capacity (see Good et al, 1966).

2 The BSA was included in order to minimise any non-specific adsorption of 2-isobutyl-3-methoxypyrazine by the test-tubes (see Section 6.4(i)a).
quickly vortexed and incubated on ice for 3 hours (see Section 6.4(iv)). The low temperature was employed in order to minimise the volatility of the radioactive pyrazine\(^1\).

Following incubation, unbound pyrazine was removed from the assay mixture by adsorption to activated charcoal, which was introduced as a slurry (0.375 ml) comprising 3% Norit GSX and Dextran (0.05 mg/ml) in 20 mM HEPES buffer, pH 7.4, containing NaCl (0.9% (w/v)); after vortexing and incubating the tubes on ice for 5 minutes, the charcoal was sedimented by centrifugation at 2000 rpm for 5 minutes in a bench centrifuge (Searle B & T centrifuge). A portion (375\(\mu\)l) of the resulting supernatant was removed from each tube, and added to 4 ml of Bray's scintillation fluid\(^2\) (Bray, 1960) contained in a plastic mini-vial. The radioactivity present in each supernatant fraction was determined by liquid scintillation counting using a Packard Prias spectrometer (53% counting efficiency for tritium). Corrections for the quenching of counting were monitored through the use of an automatic external standard.

Non-specific 2-isobutyl-3-methoxypyrazine binding was measured in parallel with the total binding, by setting up assay tubes which contained not only radioactive odorant, but also a large excess of non-radioactive 2-isobutyl-3-methoxypyrazine (6.7 \(\times\) 10\(^{-6}\)M).

For every binding experiment, a number of controls were necessary. At each concentration of \([\text{\(^3\)H}]\)pyrazine, tubes were set up containing only the assay buffer and odorant; these provided an

---

1 The high vapour pressure of this odorant necessitated all the ligand binding studies being performed in a well ventilated fume cupboard.

2 The scintillation fluid comprised butyl-PBD (7g), Triton X-100 (500 ml) and toluene (1 litre).
estimate of the adsorption efficiency of the charcoal (see Section 6.4(1)b). In addition, at various points over the concentration range employed, the total concentration of $[^3H]$pyrazine present in the incubation mixtures was determined; this provided an internal check on the loss of odorant due to test-tube adsorption during the incubation period (see Section 6.4(1)a). For these controls, 20 mM HEPEs buffer, pH 7.4, containing NaCl (0.9% w/v) was added (375 μl) to the assay tubes instead of the charcoal slurry. The radioactivity contained within these tubes was estimated as previously outlined. All assay and control tubes were prepared in at least duplicate.

When determining the concentrations of bound and free ligand, no corrections were made for the volume of charcoal present in each assay tube. However, by weighing samples of charcoal, this volume was estimated to be about 2% of the total volume of solution present. The transfer of all solutions involved in the ligand binding experiments was accomplished by the use of automatic pipettes (Gilson); their accuracy and reproducibility were periodically checked by weighing dispensed aliquots of water.
6.4 Results

The results presented graphically show experimental points which represent the mean values obtained from at least replicate assays, whilst those accumulated from several different tissue preparations indicate the mean and standard error of the individual determinations; the number of tissue preparations (n) used in each study is noted.

6.4(i) Adsorption of 2-isobutyl-3-methoxypyrazine

The proposed employment of a charcoal adsorption ligand binding assay system necessitated investigation of the adsorption characteristics of 2-isobutyl-3-methoxypyrazine.

6.4(i)a Adsorption of 2-isobutyl-3-methoxypyrazine by test-tubes

Assay tubes were set up containing $[^3\text{H}]2$-isobutyl-3-methoxypyrazine and buffer solution$^2$. After incubating on ice for 3 hours, 20 mM HEPES buffer, pH 7.4, containing 0.9° NaCl was added (375µl) to each tube; these were then vortexed and an aliquot (375µl) of each assay mixture was removed for radioactivity determination (see Section 6.3(iv)).

The results (Table 6.6) show that some $[^3\text{H}]2$-isobutyl-3-methoxypyrazine was adsorbed by each type of assay tube, irrespective of any pre-treatment. However, this adsorption was minimised by including BSA (5 mg/ml) in the assay buffer$^3$. Consequently, all the binding assays reported in this thesis were performed in Pyrex glass.

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1 Such a system has been used previously to study the binding of [16,17-$^3\text{H}$]5α-androstan-3-one to homogenates of sheep olfactory epithelium (Persaud, 1980; Persaud et al, 1980).

2 The types of buffer and assay tubes employed are shown in Table 6.6.

3 Similar findings have been reported for the adsorption to test-tubes of the urinous odorant 5α-androstan-3-one (Persaud, 1980).
Table 6.6 Adsorption of 2-isobutyl-3-methoxypyrazine by test-tubes

<table>
<thead>
<tr>
<th>Type of Tube</th>
<th>% loss of original [3H]2-isobutyl-3-methoxypyrazine (± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Polypropylene</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>b) Polypropylene scintillation mini-vial</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>c) Pyrex glass</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>d) Siliconised Pyrex glass</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>e) Pre-washed Pyrex glass</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>f) BSA-containing Pyrex glass</td>
<td>1 ± 0</td>
</tr>
</tbody>
</table>

Various assay tubes were set up containing [3H]2-isobutyl-3-methoxypyrazine (10 pmol) and

- a), b), c) 20 mM HEPES buffer, pH 7.4 (140 µl)
- d) 20 mM HEPES buffer, pH 7.4 (140 µl), following siliconisation with Sigmacote
- e) 20 mM HEPES buffer, pH 7.4 (140 µl), following overnight soaking in 10 mM HEPES buffer, pH 7.4, containing EDTA (1 mM) and BSA (5 mg/ml), and subsequent drying
- f) 10 mM HEPES buffer, pH 7.4 (140 µl), containing EDTA (1 mM) and BSA 5 mg/ml.

After incubating on ice for 3 hr., 20 mM HEPES buffer, pH 7.4, containing 0.9% NaCl was introduced (375 µl) to each tube; an aliquot (375 µl) of each assay mixture was then withdrawn for radioactivity determination.

The results indicate means ± S.E.M. for triplicate determinations.

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1 Sigmacote was introduced to the test-tubes and decanted off after 5 minutes. This operation was repeated after a 15 minute interval. The tubes were then washed with distilled water and dried in an oven. The degree of protection afforded by siliconisation against the test-tube adsorption of [3H]2-isobutyl-3-methoxypyrazine was similar to that reported previously for the odorous steroid 5α-androst-16-en-3-one (see Gower, D.B. and Hancock, M.R. (1982) in 'Olfaction and Endocr. Regul.', Proc. Eur. Chemorecept. Res. Organ. Symp., 4th 1981 (Ed. by Breipohl, W.); IRL Press Ltd., London. pp 267-277).
test-tubes and in the presence of a BSA (5 mg/ml)-containing buffer solution.

The degree of protection afforded by BSA against the test-tube adsorption of $[^{3}H]$pyrazine was found to vary considerably over a range (0 - 5 mg/ml) of albumin concentrations (Figure 6.9); at concentrations greater than 5 mg/ml, no further decrease in odorant adsorption was detected (not shown).

6.4(1)b Adsorption of 2-isobutyl-3-methoxypyrazine by charcoal

Assay tubes were set up containing BSA buffer and a range of concentrations of $[^{3}H]$2-isobutyl-3-methoxypyrazine. After incubating on ice for 3 hours, a charcoal slurry (375µl) consisting of charcoal and dextran (0.05 mg/ml) in 20 mM HEPES buffer, pH 7.4, containing 0.9% NaCl was added to each tube; these were immediately vortexed, left to stand on ice for 5 minutes, and then centrifuged at 2000 r.p.m. for 5 minutes in a bench centrifuge (Searle B & T centrifuge). An aliquot (375µl) of the resulting supernatant was withdrawn from each tube for radioactivity determination (see Section 6.3(iv)). Appropriate controls were prepared in parallel with the assay tubes.

The accompanying results show that different batches and/or concentrations of charcoal exhibited varying degrees of adsorption efficiency (Figure 6.10). Slurries containing decolourising charcoal (3% (w/v)) displayed acceptable adsorption profiles (see Figure 6.10(b); 3% decolourising charcoal(1)); the efficiency of adsorption decreased at the lower $[^{3}H]$pyrazine concentrations, but was satisfactory over a wide concentration range. Three different batches

1 The types and concentrations of charcoal employed are shown in Figure 6.10.

2 Similar findings have been reported for the adsorption by charcoal (Norit GSX) of [16,17-$^3$H] 5α-androstan-3-one (Fersaud, 1980).
Figure 6.9 Effect of bovine serum albumin (BSA) on the adsorption of 2-isobutyl-3-methoxypyrazine by test-tubes

Assay tubes were prepared containing $[^3\text{H}]2$-isobutyl-3-methoxypyrazine (10 pmol) and 10 mM HEPES buffer, pH 7.4, (140 µl) containing EDTA (1 mM) and BSA as indicated. Experiments were performed as described in the legend to Table 6.6.

The figure shows results obtained from a single experiment; it is representative of those obtained from two further studies.
Assay tubes containing a range of concentrations of [3H]2-isobutyl-3-methoxypyrazine and 10 mM Hepes buffer, pH 7.4, incorporating EDTA (1 mM) and BSA (5 mg/ml) were incubated on ice for 3 hr.

A charcoal slurry, comprising charcoal (as indicated) and dextran (0.05 mg/ml) in 20 mM Hepes buffer, pH 7.4 containing 0.9% NaCl, was then added (375 µl) to each tube. Following centrifugation, an aliquot (375 µl) of the resulting supernatant was withdrawn from each tube for radioactivity determination.

These findings, which were obtained from single experiments, are representative of those acquired from triplicate studies.
Figure 6.10 Adsorption of 2-isobutyl-3-methoxypyrazine by charcoal

(a)

[\[^{3}\text{H}2\text{-Isobutyl-3-methoxypyrazine originally present (pmol)}\]]

- $0.5\% (w/v)$ activated charcoal (untreated powder)
- $1\% (w/v)$ activated charcoal (untreated powder)
- $1\% (w/v)$ decolourising charcoal (Norit GSX)
- $3\% (w/v)$ decolourising charcoal (Norit GSX)

(b)

[\[^{3}\text{H}2\text{-Isobutyl-3-methoxypyrazine originally present (pmol)}\]]

- $3\% (w/v)$ decolourising charcoal (1) (Norit GSX)
- $3\% (w/v)$ decolourising charcoal (2) (Norit GSX)
- $6\% (w/v)$ decolourising charcoal (Norit GSX)

(□ and □ represent different batches of decolourising charcoal)
of decolourising (i.e. non-activated) charcoal were investigated, and the best was employed (as a 3% (w/v) slurry\(^1\)) in all subsequent ligand binding assays.

6.4(ii) Perception of 2-isobutyl-3-methoxypyrazine by sheep

At the outset of the present studies, it was not known whether sheep were able to perceive 2-isobutyl-3-methoxypyrazine as an odorant. Consequently, attempts were made to obtain EOGs to this compound from an in vitro sheep preparation; such an approach has been utilised previously to show that sheep can perceive the urinous odorant 5α-androstan-3-one (Persaud, 1980; Persaud et al, 1980; Dodd and Persaud, 1981).

The methodology employed has been described elsewhere (see Squirrell, 1978). Good quality EOG responses were routinely observed (Figure 6.11), thus indicating that 2-isobutyl-3-methoxypyrazine does interact with sheep olfactory epithelium.

6.4(iii) Degradation of \([^3\text{H}]2\text{-isobutyl-3-methoxypyrazine}\)

The stability of \([^3\text{H}]2\text{-isobutyl-3-methoxypyrazine}\) throughout the ligand binding assay procedure was examined by thin layer chromatography (t.l.c.).

Assay tubes containing buffer, the appropriate concentration of a 13,000 x g supernatant fraction of sheep olfactory epithelium, and \([^3\text{H}]2\text{-isobutyl-3-methoxypyrazine}\) (6.7 x 10\(^{-8}\)M) were set up in duplicate (see Section 6.3(iv)). After the addition of the tissue preparation, one of each pair of tubes was incubated on ice for 3 hours. The \([^3\text{H}]\text{pyrazine}\) in each of the remaining tubes was extracted with

---

\(^1\) Although slurries containing 6% (w/v) decolourising charcoal exhibited slightly improved adsorption characteristics, the volume occupied by this concentration of charcoal impaired the withdrawal of supernatant fluid from the assay tubes following centrifugation. Consequently, such slurries were not employed in the ligand binding assays.
Figure 6.11  The ECG response to 2-isobutyl-3-methoxypyrazine obtained from an in vitro preparation of sheep olfactory epithelium

A typical ECG response from intact sheep olfactory epithelium in response to stimulation by 2-isobutyl-3-methoxypyrazine (0.09 µM); the experimental methodology was as described by Squirrell (1978). Stimulus duration is indicated by the horizontal bar.
diethyl ether (2 x 250µl); aliquots (usually 30 - 80µl) of these extracts were then spotted onto plastic-backed silica gel (0.2 mm thick; pre-coated) t.l.c. plates (2.5 cm x 10 cm). Non-radioactive 2-isobutyl-3-methoxypyrazine (about 0.5µl) was employed as carrier ligand. The t.l.c. plates were developed, treated and analysed as previously described (see Section 6.2(ii)b). Following their incubation period on ice, the other assay tubes were similarly handled.

The accompanying results (Figure 6.12), which are representative of many similar examinations performed throughout the course of this present work, show that virtually identical chromatographic profiles were obtained at the commencement and termination of the binding assay procedure. These findings, which indicate that no degradation of the [3H]-pyrazine occurred during the assay period, confirm that the binding activity reported in this chapter reflects the interaction of genuine [3H]2-isobutyl-3-methoxypyrazine with sheep olfactory epithelium, and not the binding of odorant degradation products.

6.4(iv) Association kinetics of the ligand binding

The appropriate concentration of a 13,000 x g supernatant fraction of sheep olfactory epithelium was incubated on ice with [3H]2-isobutyl-3-methoxypyrazine (6.7 x 10^{-8}M) in the presence and absence of unlabelled pyrazine (6.7 x 10^{-6}M). At various time intervals, charcoal (in the form of a 3% slurry) was added to particular tubes and the amount of bound ligand was determined as previously described (see Section 6.3(iv)).

The results of a typical experiment (Figure 6.13) show that the time taken to reach an equilibrium was about 120 minutes for the "total" binding and about 80 minutes for the "non-specific" binding components; the "specific" binding components reached an equilibrium
Duplicate assay tubes were set up containing 10 mM HEPES buffer, pH 7.4, incorporating EDTA (1 mM) and BSA (5 mg/ml), a portion of a 13,000 x g supernatant fraction of sheep olfactory epithelium (0.1 mg) and [3H]2-isobutyl-3-methoxypyrazine (6.7 x 10^{-8} M). The [3H]pyrazine was extracted with diethyl ether (2 x 250 µl) from each pair of tubes (a) before and (b) after incubation on ice for 3 hr.

Following their loading with aliquots (30 - 80 µl) of the ether extracts, together with unlabelled carrier, thin layer chromatography plates were developed in toluene/chloroform/acetone (2 : 2 : 1), cut up into 1 cm squares and analysed in a liquid scintillation spectrometer.

The virtual superimposability of the chromatographic profiles obtained at the (a) commencement and (b) termination of the binding assay procedure indicates that the integrity of the [3H]2-isobutyl-3-methoxypyrazine is maintained throughout.

These results, obtained from a single pair of assay tubes, are representative of those found from 11 other experiments, each comprising three duplicate pairs of tubes (n = 5).
Figure 6.12  Stability of $[^3H]2$-isobutyl-3-methoxypyrazine throughout the ligand binding assay procedure

(a)  

(c.p.m. $\times 10^{-2}$)

(b)  

(c.p.m. $\times 10^{-2}$)

origin  solvent front  solvent flow
The binding of $[^{3}\text{H}]$2-isobutyl-3-methoxypyrazine ($6.7 \times 10^{-8}\text{M}$) to a $13,000 \times g$ supernatant fraction of sheep olfactory epithelium was measured in the presence and absence of unlabelled odorant ($6.7 \times 10^{-6}\text{M}$) over the periods indicated.

- Total binding
- Specific binding
- Non-specific binding

This figure, which was obtained from a single experiment, is representative of those produced by 7 further studies ($n = 6$).
after about 90 minutes. In all subsequent binding studies, the assay tubes containing ligand and tissue preparation were incubated on ice for 3 hours prior to the separation of the bound from free ligand.

6.4(v) Dissociation kinetics

The appropriate concentration of a 13,000 x g supernatant fraction of sheep olfactory epithelium and $[^3H]2$-isobutyl-3-methoxypyrazine ($6.7 \times 10^{-8} M$) were incubated together on ice for 3 hours. An aliquot (150µl) of this solution was then removed and assayed for bound ligand. To the remaining mixture was added unlabelled 2-isobutyl-3-methoxypyrazine to a final ligand concentration of $1.43 \times 10^{-5} M$. At various time intervals, further aliquots (150µl) were withdrawn and assayed for bound ligand. Parallel controls for "non-specific" binding were prepared as previously outlined (see Section 6.3(iv)).

The results obtained (Figure 6.14) show that little or no exchange between bound radiolabelled ligand and unlabelled ligand was observed over an initial period of 15 minutes. Subsequently, however, exchange did slowly occur. Additional studies have shown that the amount of "specifically"-bound radiolabelled ligand fell to about 5% of the initial value after 24 hours.

These findings indicate that no significant dissociation of the receptor-ligand complex occurred during the period required to separate bound from free ligand by charcoal adsorption.
The rate of dissociation of specifically bound ligand was measured over the periods indicated after perturbation of the equilibrium by the addition of unlabelled 2-isobutyl-3-methoxypyrazine ($1.43 \times 10^{-5}$M).

These results, which indicate the mean and range of triplicate assays from a single experiment, are typical of 3 further investigations ($n = 4$).
6.4(vi) **Affinity of binding**

6.4(vi)a **Identification of saturable binding**

A 13,000 x g supernatant fraction of sheep olfactory epithelium was incubated with a wide range of concentrations of $[^3H]2$-isobutyl-3-methoxypyrazine ($10^{-9}M - 10^{-7}M$). The bound ligand was separated from free by charcoal adsorption (see Section 6.3(iv)).

The accompanying results (Figure 6.15) are representative of four other sets of binding curves, which were obtained with different tissue preparations. All of these profiles exhibited points of inflexion, consistent with the presence of multiple binding sites possessing different affinities for 2-isobutyl-3-methoxypyrazine (Clark et al, 1979). Similar binding profiles (i.e. indicative of multiple binding sites in the picomole range) have recently been obtained for the interaction of this odorant with homogenates of cow olfactory mucosa (P. Pelosi, personal communication).

The binding studies presently reported focus upon the apparently saturable binding of high affinity which occurs within the 0 - 50 nM pyrazine concentration range (see bottom left hand corner of Figure 6.15(b)).

6.4(vi)b **Investigations on the "high affinity" binding sites for 2-isobutyl-3-methoxypyrazine**

Aliquots of a 13,000 x g supernatant fraction of sheep olfactory epithelium were incubated with $[^3H]2$-isobutyl-3-methoxypyrazine (0 - 50 nM) in the presence and absence of unlabelled odorant ($6.7 \times 10^{-6}M$). The separation of bound from free ligand was performed as previously described (see Section 6.3(iv)).

The binding curves obtained from three separate experiments are shown in Figures 6.16 - 6.18; Figures 6.16 and 6.17 illustrate the findings obtained from a single tissue preparation, whilst Figure 6.18 shows the profiles obtained from an experiment on a different
Figure 6.15 The relationship between $[^3H]2$-isobutyl-3-methoxypyrazine concentration ($10^{-9}$ - $10^{-7}$M) and total binding to a 13,000 x g supernatant fraction of sheep olfactory epithelium

A 13,000 x g supernatant fraction of sheep olfactory epithelium (0.1 mg) was incubated with a wide range of $[^3H]2$-isobutyl-3-methoxypyrazine concentrations for 3 hr. on ice prior to the separation of bound from free ligand.

(a) Concentration range: $1.6 \times 10^{-9}$M - $4.0 \times 10^{-7}$M.
(b) Concentration range: $1.6 \times 10^{-9}$M - $1.3 \times 10^{-7}$M.

Figures (a) and (b), which were obtained from single experiments, are representative of 4 other sets of binding curves acquired from different tissue preparations ($n = 5$).
The relationship between \([3^H]2\)-isobutyl-3-methoxypyrazine concentration \((10^{-9} - 10^{-7}\text{M})\) and total binding to a 13,000 x g supernatant fraction of sheep olfactory epithelium.
Figures 6.16 - 6.18

Binding of $^{3}H_{2}$-isobutyl-3-methoxypyrazine to 13,000 x g supernatant fractions of sheep olfactory epithelium

$^{3}H_{2}$-Isobutyl-3-methoxypyrazine ($0 - 5 \times 10^{-8} M$) was incubated with aliquots (0.1 mg) of a 13,000 x g supernatant fraction of sheep olfactory epithelium for 3 hr. on ice in the presence and absence of unlabelled odorant ($6.7 \times 10^{-6} M$) prior to the separation of bound from free ligand by the charcoal adsorption method.

(a)  
- Total binding
  - Non-specific binding

(b)  
- Specific binding, obtained from the data shown in (a) by subtracting the non-specific from the total binding

(c)  
- Scatchard plot of the specific binding curve shown in (b).
Figure 6.16 Binding of \([^3H]2\)-isobutyl-3-methoxypyrazine to 13,000 x g supernatant fractions of sheep olfactory epithelium

(a) Total binding

(b) Specific binding

(c) Scatchard plot

\[ n = 0.83 \text{ pmol/mg of protein} \]
\[ K_D = 1.74 \times 10^{-8} \text{M} \]
Figure 6.17  Binding of $[^3H]2$-isobutyl-$3$-methoxypyrazine to $13,000 \times g$ supernatant fractions of sheep olfactory epithelium

(a)  
Bound $\times 10^{10}$M

Total binding

Non-specific binding

Free $\times 10^8$M

(b)  
Bound $\times 10^{10}$M

Specific binding

Free $\times 10^8$M

(c)  
$\frac{\text{Bound}}{\text{Free}}$

$n = 0.85 \text{ pmol/mg of protein}$

$k_D = 1.69 \times 10^{-8}$

Scatchard plot

Bound $\times 10^{10}$M
Figure 6.18  Binding of $[^3H]2$-isobutyl-3-methoxypyrazine to 13,000 x g supernatant fractions of sheep olfactory epithelium

(a)  
Bound \( \times 10^{10}M \)

Total binding

Non-specific binding

Free \( \times 10^8M \)

(b)  
Bound \( \times 10^{10}M \)

Specific binding

Free \( \times 10^8M \)

(c)  
\[ \frac{\text{Bound}}{\text{Free}} \]

\[ n = 0.85 \text{ pmol/mg of protein} \]

\[ k_D = 1.69 \times 10^{-6}M \]

Scatchard plot
preparation. These results are representative of investigations performed in at least duplicate on nine separate tissue preparations; each preparation consisted of the pooled olfactory epithelium from three or four sheep.

The results show that over a ligand concentration range of 0 - 7.5 pmoles, saturable binding curves were obtained for the specific interaction of $[3\text{H}]2$-isobutyl-3-methoxypyrazine with 13,000 x g supernatant fractions of sheep olfactory epithelium. A large amount of non-specific binding was observed with each of the tissue preparations examined\textsuperscript{1}.

Scatchard analysis (Figures 6.16(c) - 6.18(c)) of the specific binding curves gave straight-line plots indicative of a single type of binding site (Titeler, 1961). The total number of binding sites (n) and the dissociation constant (KD) for the binding interaction (mean ± S.E.M. of 21 separate experiments; n = 9) were calculated to be 0.86 ± 0.05 pmoles/mg of protein and $(1.68 ± 0.12) \times 10^{-8}$M respectively.

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\textsuperscript{1} Similar amounts of non-specific binding have been previously reported for the binding of [16,17 - $^3\text{H}]5\alpha$-androstan-3-one to sheep olfactory epithelium (Persaud, 1980; Persaud \textit{et al}, 1980; Dodd and Persaud, 1981).
6.4(vii) Control experiments

Ligand binding assays were performed (see Section 6.3(iv)) in which 13,000 x g supernatant fractions of sheep olfactory epithelium were replaced by equivalent fractions of sheep respiratory epithelium; brain and liver, and by the total olfactory mucosa homogenate (i.e. the 600 x g supernatant fraction; see Section 6.3(i)); studies were also carried out on pre-heated samples (80°C for 20 minutes) of 13,000 x g olfactory supernatant fractions.

Experiments were performed in at least triplicate on three separate preparations of each tissue fraction. Representative results (Figures 6.19 - 6.23) obtained from single binding experiments show that no specific binding of \[^{3}H\]2-isobutyl-3-methoxypyrazine was detected with any of the tissue preparations examined\(^1\). The observed localisation of specific binding components in the olfactory mucosa fulfils one of the criteria for the identification of an odorant receptor site (see Table 6.1; Quatrecasas and Hollenberg, 1976; Titeler, 1981).

\(^1\) The pre-heating of olfactory supernatant fractions produced coagulation of the tissue preparation.
[\[^3\text{H}\]2-Isobutyl-3-methoxypyrazine (0.5 \times 10^{-8} \text{M}) was incubated with aliquots (0.1 mg) of the appropriate tissue fraction for 3 hr. on ice in the presence and absence of unlabelled odorant (6.7 \times 10^{-6} \text{M}) before separating the bound from free ligand by charcoal adsorption.
Figure 6.19 Binding of $[^{3}H]2$-isobutyl-$3$-methoxypyrazine to 13,000 x g supernatant fractions of sheep respiratory epithelium

(a) Total binding
   o Non-specific binding

(b) Scatchard plot of the total binding data shown in (a)

These results are representative of those obtained from 9 other binding experiments ($n = 3$)
Figure 6.20  Binding of $[^3H]$2-isobutyl-3-methoxypyrazine to 13,000 x g supernatant fractions of sheep brain

This figure is typical of the findings obtained from 8 other binding experiments (n = 3)
Figure 6.21 Binding of $[{}^3\text{H}]$2-isobutyl-3-methoxypyrazine to 13,000 x g supernatant fractions of sheep liver

(a) Total binding
   o Non-specific binding

(b) Scatchard plot of the total binding data shown in (a)

These findings are typical of those obtained from 9 other binding experiments ($n = 3$)
Figure 6.22 Binding of $^{3}\text{H}2$-isobutyl-3-methoxypyrazine to pre-heated samples of 13,000 x g supernatant fractions of sheep olfactory epithelium

This figure is representative of the findings obtained from 8 other binding experiments ($n = 3$)
Figure 6.23

Binding of $[^3H]2$-isobutyl-3-methoxypyrazine to 600 x g supernatant fractions of sheep olfactory epithelium

(a) 

<table>
<thead>
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<th>Bound x $10^{10}$M</th>
<th>Free x $10^{8}$M</th>
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</thead>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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<tr>
<td>5</td>
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</tbody>
</table>

(b) 

<table>
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<th>Bound/Free</th>
<th>Bound x $10^{10}$M</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0</td>
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<td>1.0</td>
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<tr>
<td>0.015</td>
<td>2.0</td>
</tr>
</tbody>
</table>

(a) • Total binding
   ○ Non-specific binding

(b) ○ Scatchard plot of the total binding data shown in (a)

These results are typical of those obtained from 8 other binding experiments (n = 3)
6.4(viii) Effect of solvent conditions on the binding of \[^{3}H\]2-isobutyl-3-methoxypyrazine to 13,000 x g supernatant fractions of sheep olfactory epithelium

Ligand binding assays were performed (see Section 6.3(iv)) under a variety of solvent conditions (i.e. at pH 2.0 and pH 11.0, and in the presence of ethanol (15%(v/v)) or SDS (0.1% (w/v)). All experiments were carried out on three separate tissue preparations; standard assays at pH 7.4 provided internal controls for these studies.

The results obtained (Figure 6.24) show that specific binding activity was abolished under extreme solvent conditions\(^1\). Proteolytic treatment of the sensory tissue has been previously reported to reduce or abolish odorant binding; these findings, together with those obtained from electrophysiological studies employing sulphydryl reagents (e.g. see Getchell, 1971; Getchell and Gesteland, 1972; Menevse, 1977; Menevse et al, 1977b, 1978; Shirley et al, 1980, 1981, 1983a), have been interpreted as evidence for the protein nature of olfactory receptor sites (e.g. see Fesenko et al, 1979; Felosi et al, 1981).

6.4(ix) Gel chromatography

Columns of Sephacryl S200 Superfine (0.9 x 30 cm) were calibrated for molecular weight estimation using bovine serum albumin (BSA), adenosine deaminase and myoglobin as standards.

Ligand binding assays were set up as previously described (see Section 6.3(iv)). Following the incubation on ice for 3 hours of a 13,000 x g supernatant fraction of sheep olfactory epithelium (about 1.5 mg/ml) with \[^{3}H\]2-isobutyl-3-methoxypyrazine (3.33 x 10^-8M), the pooled mixture from several assay tubes was applied to a gel filtration column previously equilibrated with 10 mM HEPES buffer, pH 7.4, containing EDTA (1 mM) and was eluted with this same buffer solution.

\(^1\) The detection of some binding activity under all conditions is consistent with the previously noted ability of this pyrazine odorant to bind or adsorb to a variety of surfaces (see Sections 6.4(i) and 6.4(vii)).
Figure 6.24  Effect of solvent conditions on the binding of [3H]2-isobutyl-3-methoxypyrazine to 13,000 x g supernatant fractions of sheep olfactory epithelium

Assay tubes were set up containing [3H]2-isobutyl-3-methoxypyrazine (3.33 x 10^{-8} M), aliquots (0.1 mg) of a 13,000 x g supernatant fraction of sheep olfactory epithelium, and

A  10 mM HEPES buffer, pH 7.4, containing EDTA (1 mM) and BSA (5 mg/ml)

B  10 mM HEPES buffer, pH 0.6, containing EDTA (1 mM) and BSA (5 mg/ml) (pH 2.0 in assay)

C  10 mM HEPES buffer, pH 12.5, containing EDTA (1 mM) and BSA (5 mg/ml) (pH 11.0 in assay)

D  10 mM HEPES buffer, pH 7.4, containing EDTA (1 mM), BSA (5 mg/ml) and ethanol (55% (v/v)) (15% EtOH in assay)

E  10 mM HEPES buffer, pH 7.4, containing EDTA (1 mM), BSA (5 mg/ml) and SDS (0.37% (w/v)) (0.1% SDS in assay)

Following their incubation on ice for 3 hr., in the presence and absence of unlabelled 2-isobutyl-3-methoxypyrazine (6.7 x 10^{-6} M), the separation of bound from free ligand was achieved by the charcoal adsorption method.

These results show the means and, where appropriate, the ranges of values obtained from 9 separate experiments performed under each solvent condition (n = 3) for the total (t) and specific (s) binding detected relative to that observed under standard assay conditions (A).
Figure 6.24  Effect of solvent conditions on the binding of $[^3H]2$-isobutyl-3-methoxypyrazine to 13,000 x g supernatant fractions of sheep olfactory epithelium
The column eluate was collected in fractions (25 drops; about 0.54 ml), aliquots of which were withdrawn for estimation of radioactivity (see Section 6.3(iv)) and protein (see Section 6.3(iii)) content.

Figure 6.25 shows the elution profiles obtained from two investigations on separate tissue preparations; they are representative of those obtained from other experiments involving four different preparations. Four relatively sharp peaks of radioactivity were observed in fractions possessing estimated molecular weights of 77,000, 55,000, 32,000 and 23,000. These were followed by a single large peak of free (i.e. unbound) $[^3H]2$-isobutyl-3-methoxypyrazine (not shown).

The results obtained are consistent with those reported for the chromatographic fractionation of $[^3H]2$-isobutyl-3-methoxypyrazine-bound cow olfactory mucosa homogenate (Figure 6.26; Pelosi et al, 1981).

6.4(x) Competitive binding studies

The appropriate concentration of a 13,000 x g supernatant fraction of sheep olfactory epithelium was incubated on ice for 3 hours with $[^3H]2$-isobutyl-3-methoxypyrazine in the presence of various unlabelled odorants at 10-, 100- and, where possible, 1000-1 times the concentration of the radioactive odorant. The separation of bound from free ligand was carried out as previously outlined (see Section 6.3(iv)).

The experiments with each non-radioactive odorant were performed in triplicate on at least three separate tissue preparations (Figure 6.27; Table 6.7). For each investigation, assay tubes were also prepared.

1 The limited solubility of some of the odorants precluded investigation at 1000-times the concentration of the $[^3H]$pyrazine.
Aliquots of a 13,000 x g supernatant fraction of sheep olfactory epithelium (1.5 mg/ml) were incubated with $[^3H]2$-isobutyl-3-methoxypyrazine (3.33 x $10^{-8}$M) for 3 hr. on ice. Subsequently, the pooled mixture from several assay tubes was loaded onto a Sephacryl S200 Superfine column (0.9 x 30 cm) and eluted with 10 mM HEPES buffer, pH 7.4, containing EDTA (1 mM).

- radioactivity eluted
- protein concentration

The results shown are from single experiments performed on two separate tissue preparations; they are representative of those obtained from 6 other experiments (n = 4).
Figure 6.25 Elution profiles from Sephacryl S200 Superfine columns of [3H]-isobutyl-3-methoxypyrazine binding by 13,000 x g supernatant fractions of sheep olfactory epithelium.

(a) 

(b)
Figure 6.26  Elution profile from a Sephadex G-100 column of [3H]-2-isobutyl-3-methoxypyrazine binding by cow olfactory mucosa homogenate

Gel chromatography on Sephadex G-100 of 300 µl of crude homogenate incubated with 50 pmoles of the radioactive pyrazine. BSA: bovine serum albumin; ADA: adenosine deaminase; MYO: sperm whale myoglobin.

Taken from Pelosi et al (1981)
[\textsuperscript{3}H] 2-Isobutyl-3-methoxypyrazine (3.33 x 10\textsuperscript{-8}M) was incubated with aliquots (0.1 mg) of a 13,000 x g supernatant fraction of sheep olfactory epithelium for 3 hr. on ice in the presence of various unlabelled odorants ((3.33x) 10\textsuperscript{-7}M; 10\textsuperscript{-6}M; 10\textsuperscript{-5}M) prior to the separation of bound from free ligand by the charcoal adsorption method. These results indicate the means \pm S.E.M. for values obtained from 9 (or 12\textsuperscript{M}) separate experiments performed with each of the non-radioactive odorants (n = 3 (or 4\textsuperscript{M})) for the % inhibition of [\textsuperscript{3}H] 2-isobutyl-3-methoxypyrazine binding.

\textsuperscript{M} 4-Butyl-5-propylthiazole, (-) carvone and (+) carvone were each employed in 12 investigations (n = 4)
(a) Inhibition of \[^{3}H\]pyrazine binding (%)
- methylpyrazine
- 2-methyl-3-methoxypyrazine
- 2-isopropyl-3-methoxypyrazine
- 2-isobutyl-3-methoxypyrazine

ratio of concentrations of competing odorant: \[^{3}H\]pyrazine

(b) Inhibition of \[^{3}H\]pyrazine binding (%)
- camphor
- patchouli oil
- menthone
- 4-butyl-5-propylthiozole

ratio of concentrations of competing odorant: \[^{3}H\]pyrazine

c) Inhibition of \[^{3}H\]pyrazine binding (%)
- n-octanethiol
- decanal

ratio of concentrations of competing odorant: \[^{3}H\]pyrazine

d) Inhibition of \[^{3}H\]pyrazine binding (%)
- 1,8-cineole
- (+)carvone
- (-)carvone

ratio of concentrations of competing odorant: \[^{3}H\]pyrazine

e) Inhibition of \[^{3}H\]pyrazine binding (%)
- trimethylamine
- isoamyl acetate

ratio of concentrations of competing odorant: \[^{3}H\]pyrazine

(f) Inhibition of \[^{3}H\]pyrazine binding (%)
- isovaleric acid
- diacetyl

ratio of concentrations of competing odorant: \[^{3}H\]pyrazine
Table 6.7  Competitive binding experiments: rank order of inhibition

Tabular representation of some of the data presented in Figure 6.27 showing the mean ± S.E.M. values of the inhibition of $[\text{H}]^{2}$-isobutyl-3-methoxy-pyrazine binding to 13,000 × g supernatant fractions of sheep olfactory epithelium (expressed as a % of the original level) obtained with a concentration ratio of competing odorant : $[\text{H}]$pyrazine of 100 : 1.
Table 6.7  Competitive binding experiments: rank
corbyterian of inhibition

<table>
<thead>
<tr>
<th>Competing odorant</th>
<th>Inhibition (%) of $[^3H]2$-isobutyl-3-methoxyrazine (3.33 x 10^{-6}M) binding in the presence of competing odorant (3.33 x 10^{-6}M) (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-butyl-5-propylthiazole</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>(-) carvone</td>
<td>67 ± 3</td>
</tr>
<tr>
<td>2-isobutyl-3-methoxyrazine</td>
<td>57 ± 2</td>
</tr>
<tr>
<td>2-isopropyl-3-methoxyrazine</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>(+) carvone</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>menthone</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>patchouli oil</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>decanal</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>2-methyl-3-methylpyrazine</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>diacetyl</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>isovaleric acid</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>isoamyl acetate</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>trimethylamine</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>octanethirol</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>camphor</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>2-methylpyrazine</td>
<td>-1 ± 1</td>
</tr>
</tbody>
</table>
containing unlabelled 2-isobutyl-3-methoxypyrazine (3.33 \times 10^{-6} M); this routine measure of non-specific binding provided an internal check on the viability of the tissue preparation under examination.

The rank order of inhibition obtained with a 100-fold excess of the test odorants (Table 6.7) shows that 4-butyl-5-propylthiazole, (-)carvone and 2-isobutyl-3-methoxypyrazine markedly affected the binding of $[^3H]2$-isobutyl-3-methoxypyrazine, whilst those stimuli positioned below 2-isopropyl-3-methoxypyrazine (i.e. (+)carvone... to...2-methylpyrazine) did not compete appreciably for the $[^3H]$pyrazine receptor sites; 2-isopropyl-3-methoxypyrazine itself behaved in a somewhat intermediate manner.

The reference odorant, 2-isobutyl-3-methoxypyrazine, is resembled, both in structure and in odour quality, by 2-isopropyl-3-methoxypyrazine (olfactory threshold: 0.002 p.p.b.; Seifert et al, 1970), whilst 4-butyl-5-propylthiazole, despite being a thiazole derivative, also possesses an odour resembling bell peppers (olfactory threshold: 0.003 p.p.b.; Buttery et al, 1976). Conversely, however, (-)carvone exhibits a spearmint-like odour (olfactory threshold: 2 p.p.b.; Friedman and Miller, 1971; Russell and Hills, 1971; Leitereg et al, 1971 a,b). Consequently it is difficult to understand the extent of competition displayed by this odorant; such a detailed explanation must await the performance of further studies. At present, it is only possible to speculate that this behaviour somehow reflects the molecular configuration of (-)carvone; considerably less competition was observed with (+)carvone\(^1\), which possesses a caraway-like odour (olfactory threshold: 8.5 p.p.b.; Friedman and Miller, 1971; Russell and Hills, 1971; Leitereg et al, 1971 a,b).

\(^1\) Recent studies on odour discrimination in the frog have found that enantiomeric forms of citronellol and carvone are discriminated by several receptor cells, mainly in quantitative terms (Revial et al, 1982; also see Duchamp, 1982).
The apparent lack of competition exhibited by 2-methyl-3-methoxypyrazine and by 2-methylpyrazine is more readily accountable; although these compounds bear structural similarities to the reference derivative, their odours differ in quality, as well as intensity, from the bell-pepper-like odorant. 2-Methylpyrazine has been described as possessing a nutty odour (olfactory threshold: 105,000 p.p.b.; Koehler et al, 1971; Pittet and Hruza, 1971), whilst the odour of 2-methyl-3-methoxypyrazine has been characterised as nutty/earthy (olfactory threshold: 4 p.p.b.; Seifert et al, 1970; Parliment and Epstein, 1973).

The results obtained throughout the course of this study are consistent with those reported by Pelosi et al, (1982) for the competitive binding of some of these ligands to cow olfactory mucosa homogenate (Figure 6.28).
Figure 6.28  Competitive binding of various ligands to cow olfactory mucosa homocenate.

Symbols: ▲, 2-methylpyrazine; ○, 2-methoxy-3-methylpyrazine; △, 2-isopropyl-3-methoxypyrazine; ◇, 2-isobutyl-3-methoxypyrazine; □, 4-butyl-5-propylthiazole.

Crude extract (0.1 ml) was incubated with [3H]2-isobutyl-3-methoxypyrazine (15 μmol) for 5 min. at 4°C in the presence of the appropriate non-radioactive odorant. The preparation of bound from free ligand was achieved by fractionating the incubated mixture on a Sephadex G-25 column.

Taken from Pelosi et al (1982)
6.5 Discussion

The results presented in this chapter indicate the presence of putative olfactory receptor sites in 13,000 x g supernatant fractions of sheep olfactory epithelium which exhibit saturable, high-affinity binding of $[^3H]2$-isobutyl-3-methoxypyrazine. Such binding was not observed with corresponding fractions of sheep respiratory epithelium, liver or brain tissue.

The sensitivity of these studies was impaired by the presence of high levels of non-specific binding (also see Persaud, 1980; Persaud et al, 1980) and by the comparatively low specific radioactivity (2.05 Ci/m mole) of the $[^3H]pyrazine; several previous ligand binding studies on the vertebrate olfactory system have employed ligands of greater specific radioactivity (e.g. see Cagan and Zeiger, 1978; Novoselov et al, 1980; Persaud, 1980; Rhein and Cagan, 1980). Consequently, the present investigations demanded that extreme care be exercised throughout the entire assay procedure; all solutions were freshly prepared prior to experimentation, and the apparatus used (e.g. automatic pipettes, liquid scintillation counter etc.) was periodically checked for accuracy and reproducibility.

The binding experiments were performed mainly on 13,000 x g supernatant fractions of sheep olfactory epithelium; such tissue separation permitted the detection of specific binding activity, which could not be observed with whole homogenate preparations (see Figure 6.23). Previous attempts to prepare pure plasma membrane preparations of the neuronal cells have proved largely unsuccessful (see Koyama et al, 1971; Menco et al, 1974; Menevse, 1977; Menco, 1977a; also see Rhein and Cagan, 1981; Hirsch and Margolis, 1981). The employment of supernatant tissue preparations afforded direct comparison with earlier studies on the binding of $[^3H]16,17$-androstan-3-one to sheep olfactory
epithelium (Persaud, 1980; Persaud et al., 1980) and with examinations of the binding of \[^{3}H\]2-isobutyl-3-methoxypyrazine to cow olfactory mucosa homogenate (Felosi et al., 1980, 1981, 1982).

The kinetics observed (see Sections 6.4(iv) and 6.4(v)) for the interaction of \[^{3}H\]2-isobutyl-3-methoxypyrazine with 13,000 x g supernatant fractions of sheep olfactory epithelium were similar to those described previously for the equivalent binding of 5α-[16,17-\(^{3}H\)]androstan-3-one (Persaud, 1980) and for the binding of odorant amino acids to an isolated olfactory preparation from rainbow trout (Cagan and Zeiger, 1978; but see Brown and Hara, 1981). However, the studies of \[^{3}H\]pyrazine binding to cow olfactory mucosa homogenate (Felosi et al., 1982), which utilised higher assay concentrations of both the tissue preparation and the tritiated ligand than the present examinations (P. Pelosi, personal communication), employed an incubation period of only 5 minutes at 4°C prior to the separation of bound from free ligand by gel filtration on a Sephadex G-25 column.

The odorant binding parameters (i.e. dissociation constants; number of binding sites of olfactory receptors) obtained previously appear to fall into two main groups, even though they have been determined with different ligands, animals, and techniques (Pelosi et al., 1982; see Table 6.3); values of \(K_D\) in the nanomolar range and a low number of binding sites (n) have been reported by Gennings et al. (1977) (\(K_D = 1.2 \times 10^{-9}\) M; \(n = 3.3\) pmol of 5α-androst-16-en-3-one/mg of protein in the sow) and by Fesenko et al. (1978, 1979) (\(K_D = 0.13 \times 10^{-9}\) M; \(n = 0.18\) pmol of L-alanine/mg of protein in the skate: \(K_D = 1 \times 10^{-9}\) M for camphor in the frog and rat), whilst values of \(K_D\) in the micromolar range and a high number of binding sites have been detected by Pelosi et al. (1978) (\(K_D = 0.6 \times 10^{-7}\) M; \(n = 32\) pmol of
5α-androstan-3-one/mg of protein in the cow and rabbit), by Persaud (1980) ($K_D = 0.4 \times 10^{-7}\text{M}$; $n = 28$ pmol of 5α-androstan-3-one/mg of protein in the cow) and by Cagan and Zeiger (1978) ($K_D = 5.6 \times 10^{-6}\text{M}$; $n = 440$ pmol of L-alanine/mg of protein in the trout). It has been suggested that these data support "the existence of two classes of binding sites in olfactory receptors" (Pelosi et al., 1982). Mason and Morton (1984) have recently probed the possibility of fast and loose binding \(^1\) in the olfactory epithelium of air-breathing vertebrates, proposing that ketones are bound as Schiff bases (just as retinal is bound in visual pigment). Employing the Schiff base-forming bacterial enzyme acetoacetate decarboxylase (AAD) as a model system, it was found that nucleophilic attack (e.g. BH\(^+_4\) reduction) of reversible AAD-carbonyl complexes produces irreversible binding to the active site in a fraction of the enzyme molecules. Treatment of the olfactory epithelium of experimental animals with solutions of cyclohexanone or ethyl acetoacetate was considered to involve a similar pathway, in which Schiff base linkages are attacked \textit{in vivo} by some endogenous nucleophile. The development of a behavioural assay for olfactory receptor inactivation, which has been used to detect a chemically-produced selective anosmia, "suggests that Schiff base formation may play a role in binding or transduction of ketone-containing odorants" (Morton and Mason, 1984).

The values of $n$ and $K_D$ ($n = 0.09$ pmoles/mg of protein;

\[ K_D = 1.68 \times 10^{-6}\text{M} \]

presently determined for the interaction of \(^3\text{H}\)2-isobutyl-3-methoxypyrazine with 13,000 x g supernatant fractions

\(^1\) Olfactory receptors "undoubtedly" have fast rates of association with odorant molecules. If the dissociation rate is also fast, the dissociation constant, $K_D$, will be comparatively large ($\geq 10^{-6}\text{M}$). A receptor will recover function rapidly with such fast and loose binding (Mason and Morton, 1984).
of sheep olfactory epithelium differ from those reported by Felosi et al (1982) for the binding of this odorant to cow olfactory mucosa homogenate (n = 2000 pmol/ml of homogenate, corresponding to 270 pmol/mg of protein; \( K_D = 1.2 \times 10^{-6} \text{M} \)). However, the identification of such a receptor (i.e. possessing a \( K_D \) in the micromolar range) was noted to "not exclude the presence of another site binding the same pyrazine with a much lower \( K_D \)" (Felosi et al, 1982). It is interesting to note that the non-linear Scatchard analysis of Felosi et al (1982) (see Figure 6.29) can be re-interpreted to represent a class of receptor sites possessing a \([^3H]pyrazine\) dissociation constant of about \( 2.08 \times 10^{-8} \text{M} \) (Figure 6.29; but see Light, 1984; Paul et al, 1984).

The localisation of specific binding activity in the olfactory epithelium (see Section 6.4(vii)), which was abolished under extreme conditions (see Section 6.4(viii)), satisfied one of the criteria for the identification of an odorant receptor site (see Table 6.1; Cuatrecasas and Hollenberg, 1976; Titeler, 1981). However, whilst the establishment of such binding fulfilled the aim of the present studies, it should be noted that the binding species itself has not been unequivocally identified as an olfactory receptor protein; the specific binding may in fact reflect interaction of the \([^3H]pyrazine\) with other components (e.g. olfactory marker protein; nucleotide binding protein) of the vertebrate olfactory system. Similar considerations have been applied to studies on the interaction of \( 5a-[16,17-[^3H]} \)androst-3-one with supernatant fractions of sheep olfactory epithelium (Persaud, 1980; Persaud et al, 1980), it being unclear whether "the observed binding is due to the presence of an olfactory receptor protein or a steroid hormone-binding protein" (Dodd and Persaud, 1981). These ambiguities clearly outline the difficulties and limitations inherent in the performance of ligand binding studies.
Figure 6.29 Scatchard analysis of the specific binding of $[^3H]$2-isobutyl-3-methoxypyrazine to cow olfactory mucosa homogenate

(a) $K_D = 1.2 \times 10^{-6}\text{ M}$

(b) $K_D = 2.1 \times 10^{-8}\text{ M}$

The upper figure (a) shows the Scatchard plot obtained by Pelosi et al. (1982) for the specific binding of $[^3H]$2-isobutyl-3-methoxypyrazine to cow olfactory mucosa homogenate ($K_D = 1.2 \times 10^{-6}\text{ M}$), whilst the lower figure (b) indicates its possible re-interpretation to show the existence of another class of receptors possessing a higher affinity for this odorant ($K_D = 2.1 \times 10^{-8}\text{ M}$). Both figures have been re-drawn from an original supplied by Dr. Pelosi (personal communication).
on olfactory tissue homogenates (see Price, 1981; Dodd and Persaud, 1981); such investigations ideally require the availability of purified membrane preparations. To this end, the attempted isolation and biochemical characterisation of ciliary plasma membranes from rainbow trout olfactory rosettes (Rhein and Cagan, 1980, 1981; see Section 1.3(i)) appears to be a potentially rewarding line of investigation. However, it has been noted that whilst biochemical studies of olfactory reception, relying on techniques that involve responses to odorants, have been relatively successful with aquatic vertebrates (e.g., see Rhein and Cagan, 1980, 1981; but see Brown and Hara, 1981) "which are sensitive to a limited number of odorants (mainly amino acids), and presumably have few olfactory receptor types", "amphibia and terrestrial vertebrates are capable of detecting a much wider range of chemical compounds and their olfactory receptors may have undergone a more extensive diversification" (Chen and Lancet, 1984; see Gesteland, 1971; Getchell, 1974; Price, 1981; Dodd and Persaud, 1981). Consequently, "employing ligand binding to study such a heterogeneous population of proteins would be extremely difficult" (Chen and Lancet, 1984). Further complications may ensue from degradation/metabolism of the chosen ligands. Recent studies have shown that a variety of materials, including essences, which often come into contact with the nasal mucosa can be metabolised to formaldehyde by rat nasal cytochrome P-450-dependent monooxygenases (Dahl and Hadley, 1983; also see Hadley and Dahl, 1982; Dahl et al., 1982; see Section 1.3(ii)c); dimethylanthranilate and p-methoxyacetophenone were metabolised by demethylation at rates greater than 1000 pmol formaldehyde/mg microsomal protein/min by nasal microsomes. It was noted that "almost certainly" other odorants are also
metabolised; with some exceptions, the best substrates for such metabolism tend to be those containing N-methyl groups and possessing some water solubility (Dahl and Hadley, 1983).

Chen and Lancet (1984) have recently adopted an alternative approach to olfactory receptor identification, based upon the documented simplicity of protein profiles in ciliary membranes in general (Stephens, 1977) and upon the anticipation that olfactory receptor molecules should constitute major protein components of the sensory ciliary membrane (Menco et al., 1976). Comprehensive electrophoretic mapping of the membrane proteins in frog olfactory cilia has revealed seven olfactory-specific polypeptides (see Section 5.4) whose physiological role may be determined by the application of gene cloning methods (see Early and Hood, 1981) and by immunological techniques.

In theory, olfactory receptors appear amenable to identification by chemical modification techniques (see Chapters 3 and 5). However, the present vapour-phase labelling studies demonstrated differential labelling effects rather than specific inactivation of particular classes of sites (see Chapter 3), such that "large-scale mapping experiments with many odorants would be required to identify the selectivity of proteins" (Dodd and Persaud, 1981). Consequently, ligand binding studies were employed as an alternative means of identifying olfactory receptors. In the light of the present findings, however, it seems likely that future work may benefit from utilising odorants which are responsive to both ligand binding and chemical modification studies, thereby permitting a direct comparison of the results obtained from the

---

1 The present study has indicated that negligible $[^3]H_2$-isobutyl-3-methoxypyrrole degradation occurred during the assay period (see Section 6.4(iii)).
two distinct lines of investigation; in this respect, the acetyl pyrazines appear particularly suitable.

The results obtained from the competitive binding experiments (see Section 6.4(x)) are consistent with the hypothesis that the vertebrate olfactory system comprises receptor sites capable of binding one or more odorants with different affinities (see Amoore, 1970a; Beets, 1971; Getchell and Gesteland, 1972; Polak, 1973; also see Getchell and Getchell, 1974; Gesteland, 1976; Section 3.4). The existence of a multiplicity of olfactory binding sites possessing "different but not necessarily exclusive specificities for various olfactory stimuli" has been previously suggested by studies on the binding of odorant amino acids in fish (Cagan and Zeiger, 1978). However, such investigations (also see Cancalon, 1978) have been cited as applying the criteria for receptor identification insufficiently rigorously; "a more comprehensive study" of amino acid accumulation by the sedimentable preparation from the olfactory rosettes of rainbow trout has demonstrated that the "binding of odorant amino acids cannot be unequivocally classified as representing olfactory receptors" (Brown and Hara, 1981). In particular, some involvement of amino acid transport was evidenced, and inconsistencies were noted between the various aspects of amino acid accumulation and electrophysiological response.

The mode of action of chemical stimuli on receptor cells has been the subject of various hypotheses (see Section 1.3), amongst which

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1 The ubiquitous nature of amino acids was a major consideration; irrespective of their roles as sensory transmitters, the naturally occurring amino acids "would be expected to bind to numerous biological sites and using them as a ligand intensifies the problem of quantifying the small numbers of receptors present" (Brown and Hara, 1981).
was the suggestion that stimuli activate or inhibit certain key enzymes, thereby altering the metabolic state of the cell. Although this line of thinking is no longer widely followed (see Beidler, 1962), one particular enzyme (Na\(^+\)-K\(^+\)-ATPase) has received attention with respect to olfactory receptor cell stimulation\(^1\) (e.g. see Koch, 1971/1972; Koch and Desaiash, 1974; Koch and Gilliland, 1977; Koch et al, 1980; also see Section 1.3) and has been suggested as a possible site of odorant interaction in the cell membrane. The reported involvement of amino acid transport\(^2\) in the accumulation of amino acids by a sedimentable fraction obtained from the olfactory rosettes of rainbow trout (Brown and Hara, 1981) may be interpreted as yet further support for Na\(^+\)-K\(^+\)-ATPase fulfilling such a role. It is perhaps worth noting that one of the features of the allosteric membrane enzyme hypothesis for olfaction (Dodd et al, 1977; Dodd, 1978; see Section 1.3) is a receptor potential brought about by a ligand-initiated conformational change in an ion-gating protein (e.g. by "direct ligand-induced conformational change of an allosteric protomer of a membrane enzyme with intrinsic ion-gating properties, such as a Na\(^+\)-K\(^+\)-ATPase or a Ca\(^{2+}\)-ATPase" (Dodd and Fersaud, 1981)).

\(^1\) This ATPase enzyme includes a subunit that has been reported to be an ionophore (Shamoo and Ryan, 1975); since changes in the ATPase activity could reflect interactions with this subunit, it is possible to imagine "a link with the membrane potential and thus with the transduction process" (Price, 1981).

\(^2\) Na\(^+\)-K\(^+\)-ATPase is closely linked with the active transport of amino acids (see Lehninger, 1975).
Pevsner et al. (1985) have recently reported the specific and saturable binding of 2-isobutyl-3-[3H]methoxypyrazine to bovine and rat nasal epithelium; specific binding was not detected in 11 other tissues assayed. Further, a soluble pyrazine odorant binding protein that constitutes about 1% of the total soluble protein in bovine nasal epithelium was purified to apparent homogeneity: polyacrylamide gel electrophoresis showed a single band of 19,000 Da and gel filtration data suggested that the native protein was a dimer of 38,000 Da. The binding of 2-isobutyl-3-[3H]methoxypyrazine to the purified protein revealed two binding sites \( K_D = 10 \times 10^{-9} \text{M}, n = 135 \text{ pmol per mg of protein}; K_D = 3 \times 10^{-6} \text{M}, n = 25 \text{ nmol per mg of protein} \). The binding affinities of a homologous series of pyrazine odorants correlated with the human odour detection thresholds of these compounds, and this, together with the regional distribution of the protein, suggested that "the protein is a physiologically relevant olfactory receptor".
CHAPTER 7: NUCLEAR MAGNETIC RESONANCE STUDIES OF
LIGAND BINDING

7.1 Introduction

Magnetic resonance spectroscopy (of which electron spin
resonance (ESR)\(^1\) and nuclear magnetic resonance (NMR) constitute
the two forms) is well adapted to the study of molecular structure
and dynamics\(^2\). It may be readily applied to investigations of
several biological phenomena (see Knowles et al., 1976), including
the structural nature of ligand binding sites (and hence the
mechanisms by which small molecules bind to larger molecules (e.g.
inhibitor-enzyme, hapten-antibody, drug-receptor interactions etc.)
which "is particularly well suited to analysis by high resolution
NMR" (Roberts and Jardetzky, 1970).

It is beyond the scope of this thesis to provide a full and
detailed account of nuclear magnetic resonance spectroscopy.
Consequently, this section describes only those aspects of the theory
that are directly relevant to the present investigations\(^3\).

7.1(i) Theory of nuclear magnetic relaxation techniques

(see Brown et al., 1977).

Any atomic nucleus with an odd mass number possesses a net
magnetic moment, and its magnetic vector will tend to orient in a
magnetic field (Mildvan and Engle, 1972); the potential energy of such

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\(^1\) This is sometimes known as electron paramagnetic resonance (EPR) since it employs unpaired (paramagnetic) electrons.

\(^2\) An example of the potential possessed by ESR is provided by a recent study on the identification of neutral and anionic
8c-substituted flavin semiquinones in flavoproteins (Edmondson et al., 1981).

\(^3\) For a brief qualitative explanation of some of the basic concepts of NMR spectroscopy, see Appendix 4.; also see Roberts and
a nucleus will be lower if the moment is pointing in the direction of the field rather than against it (Knowles et al, 1976). Basically, an NMR spectrum is obtained by exciting atomic nuclei into a high-potential energy level and then measuring the frequency at which the nuclei lose this potential energy when the sample is located in a magnetic field of known strength (Brown et al, 1977). The rate at which each nucleus loses its energy will vary according to the efficiency with which the nucleus is "coupled" to its environment: weakly coupled nuclei will require relatively long times to relax to the ground state (i.e. to lose their potential energy) and vice versa.

The mechanisms of potential energy loss by a nucleus are defined by two relaxation times: the spin-lattice relaxation time ($T_1$) and the spin-spin relaxation time ($T_2$) (Bovey, 1969). Normally $T_1$ and $T_2$ become equal when applied to protons of small molecules in non-viscous solvents (Knowles et al, 1976).

The spin-spin relaxation times observed when a small molecule binds to a macromolecule represent an average of the relaxation times in the bound and free states if:

$$T_{2\text{bound state}} \gg T \quad \text{and} \quad \left( \frac{\pi \delta_{\text{fb}} T_{2\text{bound state}}}{2} \right) \ll 1 \quad (2)$$

where $T$ represents the lifetime of the bound state, $\delta_{\text{fb}}$ represents the chemical shift difference between the bound and free states, and $T_{2\text{bound state}}$ signifies the spin-spin relaxation time of the ligand proton when the ligand is on the macromolecule (Brown et al, 1977).

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1 For a detailed account of how nuclear relaxation rates may be measured, see Mildvan and Engle (1972).
Since
\[ \frac{1}{T_2} = \pi(\Delta \nu)^2 \] (3) (Roberts and Jardetzky, 1970).

(where $\Delta \nu$ represents the line width at half maximum peak height), the spin-spin relaxation rates ($\frac{1}{T_2}$) of the protons on a ligand molecule can be determined by simply measuring the peak widths in the spectrum. This in turn reflects the ability of that ligand to bind to a given macromolecule (Brown et al, 1977).

The "non-specific" binding of ligand molecules by membrane preparations implies that indiscriminate interaction occurs between the ligand and charged or hydrophobic regions of the membrane; any specific molecular orientation of the ligand is generally superfluous (Brown et al, 1977). Consequently, non-specific binding will have the appearance of a micro-viscosity effect\(^1\), and the proton magnetic resonance ($^1\text{HMR}$) spectrum of such a sample will exhibit a small amount of line broadening that is the same for all the resonances of the ligand.

If a ligand interacts in a "specific" manner with a receptor site on a cell membrane, "specific parts of the ligand molecule should come into contact with the receptor reproducibly while other parts of that same molecule would remain in contact with the solvent" (Brown et al, 1977). The protons of the bound ligand molecule which interact with the receptor surface rapidly lose their potential energy

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\(^1\) The proton magnetic resonance spectrum of a poorly coupled sample (i.e. small molecules dissolved in a low viscosity solvent such as water) displays uniformly narrow peaks (in agreement with equation (3)). However, an increase in solution viscosity gives rise to improved coupling, resulting in uniform broadening of all the peaks in the spectrum.
to the macromolecule via dipole-dipole coupling. Therefore, their relaxation times are very short and the resonances of these protons are too broad to detect. However, provided that the immobilisation of one functional group does not affect the relaxation rates of other protons on the ligand molecule (i.e. the ligand molecule has several chemical bonds with unrestricted rotation) then the resonances of the protons that interact with the solvent will remain narrow. In addition, the excess ligand molecules free in solution will exhibit uniformly narrow proton resonances. Consequently, if the binding is reversible with a residence time shorter than the spin-spin relaxation times of the ligand protons in the bound state, then the observed line widths are a time-weighted average\(^1\) of the line widths in the "bound" and "free" states (from equations (1) - (3)). Since the relative proportions of time spent by the ligand in the bound and free states will depend upon the ratio of ligand molecule to receptor, the line broadening observed when a receptor preparation is added to a solution of ligand must also be a function of the ratio of ligand to receptor and not a function of the absolute concentration of either\(^2\) (Brown et al, 1977).

Thus, a comparison of the differences in line width (and/or chemical shifts, which themselves reflect a change in the magnetic

---

1 If \( T_2^{\text{free}} > T > T_2^{\text{bound state}} \), then line broadening will also occur, although the observed line widths will not represent an average of the line widths in the "bound" and "free" states. However, such broadening should be distinguishable from that attributed to "specific" binding (see Fischer and Jardetzky, 1965).

2 For a detailed discussion of the study of specific molecular interactions by nuclear magnetic relaxation measurements, see Jardetzky (1964).
environment of one or more groups on binding\(^1\) (Roberts and Jardetzky, 1970)) of the various protons on the ligand molecule when the ligand is "bound" and "free" determines the steric requirements of reversible ligand/receptor recognition\(^2\). It should be noted, however, that the use of this technique alone provides information only on the steric requirements for recognition and not on the overriding mechanism of that recognition\(^3\).

Nuclear magnetic resonance studies of the binding of small molecules to proteins (prototypes of which were reported by Jardetzky et al, 1961; Fischer and Jardetzky, 1965; Jardetzky and Wade-Jardetzky, 1965\(^4\)) may be loosely divided into two groups:

(a) the recording of only the ligand spectrum permits identification of the interacting groups on the small molecule, whereas (b) the elucidation of the complete complex structure also requires the spectrum of the protein to be studied (for discussions, see Roberts and Jardetzky, 1970). The present investigations involved the recording of ligand spectra only.

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1 Studies of relaxation rates rather than chemical shifts provide the more sensitive method for examining weak binding: the change in chemical shift on binding is unlikely to exceed 100 Hz., whilst the relaxation rate of a proton on the small molecule may increase by two to three orders of magnitude. However, the direction and magnitude of the chemical shift does provide a direct indication of the magnetic environment of the protons of the ligand when bound to the protein (Roberts and Jardetzky, 1970).

2 The exhibition of both the largest change in magnetic environment and the largest decrease in motional freedom would be expected by those groups that interact directly with groups on the protein (i.e. receptor) (Roberts and Jardetzky, 1970).


4 For more recent biological applications of magnetic resonance, see Knowles et al (1976), Dwek et al (1977), Shulman (1979); also see Cohn and Reed (1982).
Proton magnetic resonance studies on the vertebrate olfactory system

The binding of L-carnosine to bovine serum albumin and to mouse nasal olfactory mucosa has been previously characterised by proton magnetic relaxation spectroscopy (Brown et al., 1977, 1979).

L-carnosine is a dipeptide (β-alanyl-L-histidine) that was discovered in muscle at the turn of the century (Gulewitsch and Amiradzibi, 1900). The presence of high levels (i.e. 1 to 20 mM) of carnosine in the skeletal muscle of several species has been well documented (Crush, 1970), although its function in muscle tissue is still unclear (Brown et al., 1977). Recent studies have shown that carnosine is present in the nasal olfactory mucosa and olfactory bulb of several mammalian species (Table 7.1) at a higher concentration (about 2 mM) than elsewhere in the mammalian central nervous system (Margolis, 1974, 1981; Neidle and Kandera, 1974; Ferriero and Margolis, 1975; Burd et al., 1982; also see Hirsch et al., 1978; Hirsch and Margolis, 1979; Nadi et al., 1980); the enzymes necessary for its synthesis and hydrolysis (see Margolis et al., 1979, 1983; Margolis, 1981) also occur in this neural pathway (Margolis, 1975; Harding and Margolis, 1976; Margolis and Grillo, 1977; also see Neidle and Kandera, 1974; Harding et al., 1977; Harding and Wright, 1979), with carnosine synthetase being present in the olfactory epithelium at higher activities than in other nervous tissue.

These observations suggested that carnosine may play some

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1 Carnosine synthetase, which has been purified 600-fold from mouse olfactory bulb, catalyses the synthesis of carnosine from β-alanine and L-histidine (see Ng et al., 1977; Horinishi et al., 1978; Ng and Marshall, 1978; Harding and O'Fallon, 1979; for review, see Margolis, 1981).

2 The turnover of olfactory carnosine is on the order of 10 - 20 hr., whilst that in muscle is on the order of 10 days (Margolis and Grillo, 1977).
<table>
<thead>
<tr>
<th>Species</th>
<th>Olfactory bulb (μmole/mg tissue)</th>
<th>Whole brain (μmole/mg tissue)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carnosine</td>
<td>Homocarnosine</td>
<td>Carnosine</td>
</tr>
<tr>
<td>Mouse</td>
<td>1.8</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>Mouse</td>
<td>0.9–1.9</td>
<td>0.04–0.12</td>
<td>—</td>
</tr>
<tr>
<td>Hamster</td>
<td>1.2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Pig</td>
<td>0.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dog</td>
<td>1.2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Gecko</td>
<td>3.9</td>
<td>—</td>
<td>0.16</td>
</tr>
<tr>
<td>Rat</td>
<td>2.7</td>
<td>—</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Taken from Margolis (1981)
physiologically-important role in olfaction (see Tonosaki and Shibuya, 1979; MacLeod and Straughan, 1979; Gonzalez and Freeman, 1979) and in support of this hypothesis, Brown et al. (1977) reported a tissue-specific binding of carnosine in the mouse nasal olfactory mucosa (as determined by proton magnetic relaxation spectroscopy). Subsequent investigations have indicated that carnosine may function as a neurotransmitter of olfactory chemoreceptor neurones (Rochel and Margolis, 1982; but see Brown et al. 1977, 1979; MacLeod and Straughan, 1979).

Proton magnetic relaxation studies of the steric and charge requirements for the binding of L-carnosine by BSA have shown that the histidinyl side chain of the dipeptide is responsible for primary recognition by the binding site (Figure 7.1; Brown et al., 1977); this recognition is specific to a particular orientation of the histidinyl side chain which is itself determined by the other amino acid (ß-alanyl) residue of the dipeptide. In addition, carnosine binds to crude particulate fractions of nasal olfactory mucosa in the same steric orientation as it does to BSA; similar binding was not observed with preparations of olfactory bulb, lung, cerebrum, kidney or muscle. The tissue-specific binding was shown to be reversible and saturable, and to occur within the physiological concentration range of carnosine (Brown et al., 1977). Subsequent studies have utilised the binding of carnosine to BSA as a model system to identify potential competitive inhibitors of carnosine binding that could ultimately be tested for activity in the olfactory pathway. The binding to BSA was found to be "a good model of non-specific binding of carnosine to tissue preparations but not of the specific binding of carnosine to the nasal olfactory epithelium" (Brown et al., 1979); as well as requiring the proper conformation of
The $^1$H NMR spectrum of 10 mM carnosine and the effect of 0.8 mM bovine serum albumin and 0.4 mM bovine gamma globulin on spectral line widths. The resonances of carnosine are assigned by the letters A-G, and the large unlabelled resonance in the middle of each spectrum arises from HOD. The spectral range shown in these spectra is 2.5 - 8.9 ppm downfield from tetramethylsilane (TMS) contained in a coaxial capillary. Minor variations in chemical shift of the imidazole ring protons, $H_A$ and $H_B$, are the result of small variations in pH and NaCl concentration.

Taken from Brown et al (1977)
the histidinyl residue, the interaction with olfactory epithelium also appears to require recognition of the β-alanyl residue and of the substituents on the imidazole ring (Brown et al, 1979).

The success of these examinations suggested that other chemicals of olfactory interest may be similarly investigated. To this end, a feasibility study was performed with several heterocyclic odorants. This chapter describes their steric requirements for protein binding as determined by nuclear magnetic relaxation techniques.
7.2 Methods

7.2(i) Odorant binding to bovine serum albumin

All odorant solutions were prepared in D$_2$O (99.8\% isotopic purity), the pH being adjusted with NaOD or DCI as required; no corrections were made for isotope effects. Aliquots of such solutions were added to known amounts of bovine serum albumin to yield protein solutions of required concentrations (expressed as \% (w/v)). Odorant samples (in the presence and absence of BSA) were then transferred to 5 mm NMR tubes and maintained at 4°C until spectra were recorded.

Spectra were obtained with continuous wave techniques on a Perkin-Elmer R34 NMR spectrometer operating at a probe temperature of 22°C. All spectra were recorded with a spectrum width of 2200 Hz, and a sweep time of 450s; appropriate spectral regions were expanded as required. TSS$^2$, contained within a coaxial capillary, was routinely employed as a reference standard.

7.2(ii) The binding of 2-isopropyl-3-methoxypyrazine to 13,000 x g supernatant fractions of sheep olfactory epithelium

Supernatant fractions of sheep olfactory epithelium were prepared in cold 20 mM HEPES buffer, pH 7.4, containing EDTA (1 mM), as previously described (see Section 6.3(i)), aliquots of which were diluted with 2-isopropyl-3-methoxypyrazine and D$_2$O to yield solutions of desired odorant (2 mM) and protein concentration (expressed as \% (w/v)); the pH was adjusted with NaOD or DCI as required. Portions of these solutions were then introduced to 5 mm NMR sample tubes and maintained at 4°C until spectra were recorded. Tubes were also set

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1 The interaction of 2-isopropyl-3-methoxypyrazine (2 mM) with BSA (see Figure 7.11) was monitored on a Brucker SFC2 NMR spectrometer; see Section 7.2(ii)).

2 3-(Trimethylsilyl)-1-propanesulphonic acid
up devoid of sheep olfactory epithelium preparations, containing only 2-isopropyl-3-methoxypyrazine (2 mM) dissolved in D_2O and the HEPES buffer solution.

Spectra were obtained with pulse-Fourier transform techniques on a Bruker SFO2 NMR spectrometer, employing solvent suppression and ambient probe temperature (about 25°C). Each spectrum, comprising about 400 scans with an acquisition time of 4.09S, was recorded with a sweep width of 4000 Hz and a resolution of 0.244 Hz per point on the plot. TMS was employed as a reference standard.

7.2(iii) Interpretation of NMR spectra

The ability of the various ligands to bind to bovine serum albumin and to 13,000 x g supernatant fractions of sheep olfactory epithelium, together with the steric requirements for such interactions, were determined by comparing the linewidths of the proton resonances of the ligands in the 'bound' and 'free' states. In the accompanying results, \( \Delta_{\text{bound}} / \Delta_{\text{free}} \) indicates the observed change in linewidth when a particular ligand is mixed with protein in solution (ie 'bound') relative to when it alone is dissolved in solvent (ie 'free').
7.3 Results

The accompanying results, which are summarised in Table 7.2, show representative data obtained from single experiments; the number of experiments (n) performed in each study is indicated.

7.3(i) The binding of penicillin G to bovine serum albumin

The protein binding of penicillin (see Chow and McKee, 1945; Tompsett et al, 1947) has been examined previously using nuclear magnetic relaxation techniques and shown to primarily involve the aromatic portion of the drug molecule (Fischer and Jardetzky, 1965). Accordingly, the present studies initially sought to reproduce these observations in order to provide a methodological control for future investigations.

The proton magnetic resonance spectrum of sodium penicillin G (0.1 M) in deuterium oxide is shown in Figure 7.2, along with the effect of albumin on peak line width; all the spectral lines undergo broadening, but to varying degrees. As noted by Fischer and Jardetzky (1965) "the possibility exists that the observed broadening is non-specific rather than an indication of a drug-protein complex". However, several alternative broadening mechanisms (such as an increase in solution viscosity\(^1\), an increase in penicillin-penicillin intramolecular interactions upon addition of protein, and direct intermolecular relaxation by the protein even in the absence of complex formation) have been discredited previously (Fischer and Jardetzky, 1965) and, where feasible, during the course of this present work.

The results of a typical investigation into the effects upon the penicillin spectrum of adding albumin to the drug solution are shown

---

\(^1\) It has been previously reported that the viscosity of a solution of albumin (5% (w/v)) in D\(_2\)O is only 1.25 times as large as the viscosity of pure D\(_2\)O (Fischer and Jardetzky, 1965).
Table 7.2  Nuclear magnetic resonance studies of the steric requirements of various ligands for protein binding

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Portion of ligand molecule primarily involved in the binding to bovine serum albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>sodium penicillin G</td>
<td>phenyl ring</td>
</tr>
<tr>
<td>2-acetylpyridine</td>
<td>acetyl group</td>
</tr>
<tr>
<td>3-acetylpyridine</td>
<td>acetyl group</td>
</tr>
<tr>
<td>4-acetylpyridine</td>
<td>acetyl group</td>
</tr>
<tr>
<td>2-pyridinemethanol</td>
<td>methanolic group</td>
</tr>
<tr>
<td>3-pyridinemethanol</td>
<td>methanolic group</td>
</tr>
<tr>
<td>ethyl nicotinate</td>
<td>ethyl group</td>
</tr>
<tr>
<td>2-methylpyrazine</td>
<td>methyl group</td>
</tr>
<tr>
<td>2-methyl-3-methoxypyrazine</td>
<td>methyl and methoxy groups</td>
</tr>
<tr>
<td>2-isopropyl-3-methoxypyrazine</td>
<td>methoxy group**</td>
</tr>
<tr>
<td>2-isobutyl-3-methoxypyrazine</td>
<td>methoxy group</td>
</tr>
</tbody>
</table>

**the methoxy group is also primarily involved in the binding of 2-isopropyl-3-methoxypyrazine to 13,000 x g supernatent fractions of sheep olfactory epithelium.
Figure 7.2 Effect of bovine serum albumin on NMR spectral linewidths of sodium penicillin G

(a)

Figure 7.2 Effect of bovine serum albumin on NMR spectral linewidths of sodium penicillin G

The upper trace (a) shows portions of the $^1$HMR spectrum of sodium penicillin G (0.1M) in D$_2$O, pH 7.5, whilst the lower trace (b) indicates the effect of BSA (5% (w/v)) on these spectral regions.

(b)

These results, obtained with a fixed sodium penicillin G concentration (0.1 M; pH 7.5), show the effects of varying albumin concentrations on the linewidths of peaks 1, 4 and 5 ($n = 10$).
in Figure 7.2(c). Although peak (5) is initially the broadest peak, it is evident that the relaxation rate of peak (1) is changed by a larger factor than the relaxation rate of either peak (4) or peak (5)\(^1\) (see Fischer and Jardetzky, 1965). The preferential stabilisation of the aromatic portion of the penicillin molecule is inferred from this relative increment, rather than from the absolute extent of broadening (Jardetzky, 1964).

Figure 7.4 shows that the relaxation rate is dependent upon the penicillin-albumin ratio rather than on the albumin concentration itself; the addition of more penicillin to a given penicillin-albumin solution decreased the width of the lines. This finding is exactly opposite to what would be expected if the line broadening were due to one of the previously mentioned non-specific mechanisms (see Fischer and Jardetzky, 1965).

The attainment of proton magnetic resonance data consistent with the previously reported findings suggested that similar experimental procedures might now be applied to studies of heterocycle binding to BSA.

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\(^1\) The relative changes in the relaxation rates are consistent with the previously published data, although the absolute values of these rates differ from such findings (Fischer and Jardetzky, 1965; Figure 7.3). However, it should be noted that the spectra obtained during the course of this present work were recorded at 22°C; analysis of the previously reported studies indicates that these investigations were performed at a much higher temperature (unspecified). Consequently, the apparent differences in the two sets of results may be explained by this variation in temperature: it has been previously suggested that the fraction of albumin-bound penicillin is increased as the temperature is decreased (Fischer and Jardetzky, 1965).
Figure 7.3  Effect of bovine serum albumin on NMR spectral linewidths of potassium penicillin G

The $^1$HMR spectrum of potassium penicillin G (0.5M) in D$_2$O in the absence (upper trace) and presence (lower trace) of serum albumin (10% (w/v))

A typical example of the effect of albumin on spectral linewidth

Taken from Fischer and Jardetzky (1965)
Figure 7.4  Effect of penicillin : albumin ratio on NMR spectral linewidths of sodium penicillin G

These results, obtained with a fixed bovine serum albumin concentration (1.25% (w/v)) and three different concentrations of sodium penicillin G (10 mM; 100 mM; 200 mM) in D2O, pH 7.5, show the effects of varying penicillin : albumin ratios on the linewidths of peaks 1, 4 and 5 (see Figure 7.2) in the 1HMR spectrum of sodium penicillin G  (n = 7)
The binding to bovine serum albumin of several pyridine odorants

The steric requirements (if any) for albumin binding of 2-, 3- and 4-acetylpyridine; 2- and 3-pyridinemethanol\(^1\), and ethyl nicotinate, were investigated as previously described (see Section 7.2).

The results (Figures 7.5 - 7.7), which are typical of those obtained over a range of heterocycle concentrations (0.01 M - 0.2 M, depending upon the solubility of the individual compounds), show that the side chain groupings are primarily involved in the binding of these compounds to BSA; in all cases, the relaxation rates of the peaks corresponding to the substituent groups were changed by a larger factor than those of the aromatic proton peaks. The acetyl group peaks of the appropriate pyridine derivatives displayed the greatest rate of change; the relaxation rates of the side chain groups of ethyl nicotinate and 2- and 3-pyridinemethanol were altered to a lesser degree.

These observations suggest that the acetyl group fulfils an important role in the binding of acetylpyridines to BSA. For all the compounds examined, relaxation rates were found to be dependent upon the heterocycle-albumin ratio rather than upon the albumin concentration itself (Figure 7.8).

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\(^1\) Attempts to monitor the binding of 4-pyridinemethanol proved unsuccessful, since the peak corresponding to the methylene group became increasingly masked by the adjacent HOD peak as line broadening occurred, such that the ligand spectrum obtained in the presence of 2.5% and 5% BSA (w/v) was incompletely resolved.
Figure 7.5  Effect of bovine serum albumin on NMR spectral linewidths of 2-, 3- and 4- acetylpyridine

(a) 2-acetylpyridine (10 mM in D₂O; pH 7.2)

\[ \frac{\Delta_5 \text{ bound}}{\Delta_5 \text{ free}} = 4.5 \]
\[ \frac{\Delta_1 \text{ bound}}{\Delta_1 \text{ free}} = 2.0 \]

(b) 3-acetylpyridine (10 mM in D₂O; pH 7.2)

\[ \frac{\Delta_6 \text{ bound}}{\Delta_6 \text{ free}} = 4.3 \]
\[ \frac{\Delta_2 \text{ bound}}{\Delta_2 \text{ free}} = 1.9 \]
\[ \frac{\Delta_1 \text{ bound}}{\Delta_1 \text{ free}} = 1.8 \]

(c) 4-acetylpyridine (10 mM in D₂O; pH 7.2)

\[ \frac{\Delta_4 \text{ bound}}{\Delta_4 \text{ free}} = 4.6 \]
\[ \frac{\Delta_1 \text{ bound}}{\Delta_1 \text{ free}} = 1.6 \]
\[ \frac{\Delta_2 \text{ bound}}{\Delta_2 \text{ free}} = 1.7 \]
Figure 7.6  Effect of bovine serum albumin on NMR spectral linewidths of 2- and 3-pyridinemethanol

(a) 2-pyridinemethanol (50 mM in D$_2$O; pH 7.2)

(b) 3-pyridinemethanol (100 mM in D$_2$O; pH 7.2)

These results, obtained with fixed concentrations of (a) 2-pyridinemethanol (50 mM) and (b) 3-pyridinemethanol (100 mM), show the effects of varying albumin concentrations on the linewidths of several peaks in their $^1$H NMR spectra.
Figure 7.7  Effect of bovine serum albumin on NMR spectral linewidths of ethyl nicotinate

(a)

(b)

The upper trace (a) shows sections of the $^1$H NMR spectrum of ethyl nicotinate (0.1M) in D$_2$O, pH 7.4, whilst the lower trace (b) indicates the effect of BSA (5% (w/v)) on these spectral regions.
These results, obtained with a fixed concentration of ethyl nicotinate (0.1M; pH 7.4), show the effects of varying albumin concentrations on the linewidths of peaks 1, 4 and 7 (n = 9)
These results, obtained with a fixed bovine serum albumin concentration (1.25% (w/v)) and three different concentrations of ethyl nicotinate (10 mM; 100 mM; 200 mM) in D2O, pH 7.4, show the effects of varying ethyl nicotinate : albumin ratios on the linewidths of peaks 1 and 7 (see Figure 7.7) in the $^1$HMR spectrum of ethyl nicotinate (n = 6)
7.3(iii) The binding to bovine serum albumin of several pyrazine odorants

The steric requirements for the binding to BSA of 2-methylpyrazine, 2-methyl-3-methoxypyrazine, 2-isopropyl-3-methoxypyrazine, and 2-isobutyl-3-methoxypyrazine were examined as previously described (see Section 7.2).

The representative results\(^1\) (Figures 7.9 - 7.12) show that the relaxation rates of the peaks corresponding to substituent groups were altered by a larger factor than those of the aromatic proton peaks. In particular, the albumin-binding of 2-methylpyrazine, 2-isopropyl-3-methoxypyrazine and 2-isobutyl-3-methoxypyrazine was found to primarily involve the methyl, methoxy and methoxy groups respectively; the two substituent group peaks of 2-methyl-3-methoxypyrazine displayed similar degrees of line broadening.

The marked rates of change in the appropriate line widths suggest that the methoxy group plays an important part in the protein-binding of larger 2-alkyl-3-methoxypyrazines. The interaction of 2-isopropyl-3-methoxypyrazine with BSA was further investigated by examining the requirements for binding at reduced pyrazine and albumin concentrations\(^2\) (Figure 7.11(d)); the results obtained were consistent with the previous findings concerning the importance of the methoxy group in the protein-binding of this heterocyclic odorant.

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1 The limited solubility in D\(_2\)O of the examined pyrazines precluded investigations over a wide range of diazine concentrations.

2 Performed on a Brucker SPC2 NMR spectrometer.
Figure 7.9  Effect of bovine serum albumin on NMR spectral linewidths of 2-methylpyrazine

(a)  

Effect of bovine serum albumin on NMR spectral linewidths of 2-methylpyrazine

(b)  

The left-hand trace (a) shows parts of the $^1$HMR spectrum of 2-methylpyrazine (10 mM) in D$_2$O, pH 7.4, whilst the right-hand trace (b) indicates the effect of BSA (5% (w/v)) on these spectral regions.

(c)  

These results, obtained with a fixed concentration of 2-methylpyrazine (10 mM; pH 7.4), show the effects of varying albumin concentrations on the linewidths of peaks 1, 2 and 4 (n = 7).
The upper trace (a) shows portions of the $^1$HMR spectrum of 2-methyl-3-methoxypyrazine (10 mM) in D$_2$O, pH 7.4, whilst the lower trace (b) indicates the effect of BSA (5% (w/v)) on these spectral regions.

These results, obtained with a fixed concentration of 2-methyl-3-methoxypyrazine (10 mM; pH 7.4), show the effects of varying albumin concentrations on the linewidths of peaks 1, 3 and 4 ($n = 8$).
Figure 7.11  Effect of bovine serum albumin on NMR spectral linewidths of 2-isopropyl-3-methoxypyrazine

(a)

The upper trace (a) shows sections of the \textsuperscript{1}H NMR spectrum of 2-isopropyl-3-methoxypyrazine (10 mM) in D\textsubscript{2}O, pH 7.4, whilst the lower trace (b) indicates the effect of BSA (5% (w/v)) on these spectral regions.
Figure 7.11  Effect of bovine serum albumin on NMR spectral linewidths of 2-isopropyl-3-methoxypyrazine

(c) 2-isopropyl-3-methoxypyrazine (10 mM in D$_2$O, pH 7.4)

\[ \frac{\Delta 3 \text{ bound}}{\Delta 3 \text{ free}} = 6.6 \]
\[ \frac{\Delta 1 \text{ bound}}{\Delta 1 \text{ free}} = 2.5 \]
\[ \frac{\Delta 5 \text{ bound}}{\Delta 5 \text{ free}} = 4.5 \]

These results, obtained with fixed concentrations of 2-isopropyl-3-methoxypyrazine, show the effects of varying albumin concentrations on the linewidths of peaks 1, 3 and 5.
Figure 7.12  Effect of bovine serum albumin on NMR spectral linewidths of 2-isobutyl-3-methoxypyrazine

(a)  

\[ \begin{align*} 
1 & \text{phenyl H's} \\
3 & \text{C}_5 \text{H's} \\
6 & \text{H's of } \text{C}_1 + \text{C}_2 
\end{align*} \]

downfield from TSS

(b) 

\[ \begin{align*} 
2 & \text{ppm} \\
6 & \text{ppm} \\
9 & \text{ppm} 
\end{align*} \]

downfield from TSS

The upper trace (a) shows portions of the \( ^1 \text{HMR} \) spectrum of 2-isobutyl-3-methoxypyrazine (10 mM) in \( \text{D}_2\text{O}, \text{pH 7.4} \), whilst the lower trace (b) indicates the effect of BSA (5% (w/v)) on these spectral regions.

(c) 

\[ \begin{align*} 
\Delta_3 \text{ bound} & \quad 7.3 \\
\Delta_3 \text{ free} \\
\Delta_1 \text{ bound} & \quad 3.1 \\
\Delta_1 \text{ free} \\
\Delta_6 \text{ bound} & \quad 5.3 \\
\Delta_6 \text{ free} 
\end{align*} \]

These results, obtained with a fixed concentration of 2-isobutyl-3-methoxypyrazine (10 mM; pH 7.4), show the effects of varying albumin concentrations on the linewidths of peaks 1, 3 and 6 (n = 9).
The binding of 2-isopropyl-3-methoxypyrazine to 13,000 \( x \) g supernatant fractions of sheep olfactory epithelium

The binding of odorants to BSA may serve as a useful model system for identifying those portions of odorant molecules involved in the interaction with olfactory receptors; previous findings have suggested that such receptors are proteinaceous in nature (see Section 1.3(iii)). However, it should be noted that limitations have been reported for the model system provided by the binding of carnosine to BSA (Brown et al, 1979).

In an attempt to validate the odorant/BSA model system, nuclear magnetic relaxation techniques were employed to study the interaction of 2-isopropyl-3-methoxypyrazine\(^1\) with 13,000 \( x \) g supernatant fractions of sheep olfactory epithelium (see Section 7.2).

Figure 7.13 shows that the relaxation rate of the peak corresponding to the methoxy group was changed by a larger factor than the relaxation rate of either the aromatic or the isopropyl proton peaks. This finding, which is in agreement with the observations on the albumin-binding of 2-isopropyl-3-methoxypyrazine (see Section 7.3(iii)), demonstrates the importance of the methoxy group in the interaction of this odorant with homogenates of sheep olfactory epithelium.

\(^1\) The slightly greater solubility in \( D_2 O \) of 2-isopropyl-3-methoxypyrazine determined that this heterocycle rather than 2-isobutyl-3-methoxypyrazine be employed in the present investigations.
These results, obtained with a fixed concentration of 2-isopropyl-3-methoxypyrazine (2 mM; pH 7.4), show the effects of varying protein concentrations of 13,000 x g supernatant fractions of sheep olfactory epithelium on the linewidths of peaks 1, 3 and 5 (see Figure 7.11) in the $^1$HMR spectrum of 2-isopropyl-3-methoxypyrazine ($n = 6$).
7.4 Discussion

The results presented in this chapter have demonstrated the feasibility of using proton magnetic relaxation techniques to examine the steric requirements for protein binding of some odorants. The interaction of several pyridine (2-, 3- and 4-acetylpyridine; 2- and 3-pyridinemethanol; ethyl nicotinate) and pyrazine derivatives (2-methoxypyrazine; 2-methyl-3-methoxypyrazine; 2-isopropyl-3-methoxypyrazine; 2-isobutyl-3-methoxypyrazine) with bovine serum albumin has been shown to primarily involve a side chain grouping of the heterocycles. The limited solubility in D₂O of many heterocycles restricted the choice of compounds suitable for investigation; in particular, the employment of 4-butyl-5-propylthiazole, which possesses a potent bell pepper odour and an extremely low olfactory threshold (0.003 p.p.b.; Butterly et al., 1976; see Section 6.4(x)), was precluded.

The studies with the pyridyl compounds show that the degree of side chain involvement\(^1\) remained relatively constant for all of the heterocycles investigated (with the possible exception of 3-pyridinemethanol), although the absolute amounts of binding (as indicated by comparing the \(\Delta\text{bound}/\Delta\text{free}\) ratios for equivalent peaks of the six compounds) varied quite markedly; the 2-, 3- and 4-acetylpyridines exhibited the greatest extents of binding.

The results obtained from the examinations of pyrazine binding to albumin indicate that the relative involvements of the alkyl and the methoxy groups varied according to the nature of the former substituent; as the chain length of the alkyl group increased from methyl to isopropyl, so the relative importance of the methoxy-group\(^1\) in the

\[ \frac{\Delta\text{bound (side grp)}}{\Delta\text{free (side grp)}} = \frac{\Delta\text{bound (aromatic)}}{\Delta\text{free (aromatic)}} \]
binding interaction was found to increase. Studies on the binding of
2-isopropyl-3-methoxypyrazine to 13,000 x g supernatant fractions of
sheep olfactory epithelium supplied results consistent with those
obtained from investigations of its interaction with albumin; the
relaxation rate of the peak corresponding to the methoxy group was
changed by a larger factor than the rates of the other peaks,
indicating that this group is responsible for primary recognition by
the binding site.

These findings on the pyrazines may be usefully considered in
conjunction with previous psychophysical observations. The present
studies have shown that the extent of pyrazine binding to BSA (as
indicated by comparing the Δbound/Δfree ratios for equivalent peaks
(i.e., aromatic, alkyl, methoxy)) increased as the alkyl group of the
2-alkyl-3-methoxypyrazines varied from methyl to isobutyl, such that
the greatest amount of binding activity was observed for the albumin-
interaction of 2-isobutyl-3-methoxypyrazine. These findings appear
generally consistent with observations on the odour thresholds in water
of a series of methoxypyrazines related to 2-isobutyl-3-methoxypyrazine
(Figure 1.31; Taranishi et al., 1974; see Section 1.4(ii)); their
thresholds were noted to increase by a factor in the neighbourhood of
10^6 as the chain length of the 2-alkyl-3-methoxypyrazines was reduced
from 2-hexyl-3-methoxypyrazine to 2-methoxypyrazine. In addition, it
was found that removal of the methoxy group from 2-isobutyl-3-methoxy-
pyrazine increased the olfactory threshold by a factor of about 10^5.
This observation supports the finding that the methoxy group is
primarily involved in the protein binding of this odorant.

It has been noted previously that the size and shape of an
odorant molecule, together with the distribution of polar groups,
determine its odour type (Amoore, 1970a; Dodd, 1976), although the
structural requirements for a particular type of odour have been only
partially defined (Boelens, 1974; Beets, 1978; Chloff and Flament, 1979; see Section 1.4(i)). Some polar groups impart a distinctive odour type to an odorant series, for which it is possible to imagine odorant binding sites with complementary polar groups. The single polar groups possessed by most odorants may be used for orienting the ligand at the binding site using molecular interactions such as hydrogen bonding and electrostatic interactions which have a directional element (Dodd and Persaud, 1981). The present findings on the 2-alkyl-3-methoxypyrazines appear compatible with such ideas; besides fulfilling the primary role of site recognition and binding, the methoxy group may also function in an orientating capacity to correctly align the remainder of the odorant molecule with the receptor binding site. The correct molecular binding orientation will become more highly defined as the odorant structure becomes more highly developed. It is attractive to speculate that the degree of such orientation (i.e., the exactness of odorant alignment with the binding site; hence the affinity of binding) could form the basis of a patterned response (see Section 1.2(vi); also see Persaud and Dodd, 1982) which would constitute quality coding at the level of the primary olfactory neurones.

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1 Studies of human partial anosmia to isobutyric and isovaleric acid have concluded that there is, in the normal nose, a detector "specialised to react to the carboxylic acid functional group" (see Davies, 1971; also see Section 5.4).
CHAPTER 8: GENERAL DISCUSSION AND CONCLUDING REMARKS

For the first time, attempts have been made to investigate the quality coding mechanism which operates for certain heterocyclic odorants at the level of the primary olfactory neurones; a variety of electrophysiological (Chapters 3, 4 and 5), biochemical (Chapter 6) and biophysical (Chapter 7) techniques have furnished concrete results which may serve as pointers for future studies. The findings suggest that the vertebrate olfactory system comprises several sets of receptor proteins with relatively broad binding specificities (see Beets, 1971; Polak, 1973; Getchell and Getchell, 1974); in addition, it is postulated that the receptor sites directed against sweaty-smelling fatty acids (see Chapter 5) and "green" heterocycles (see Chapter 7) are able to recognise appropriate functional groups (i.e. the carboxylate anion and the methoxy group respectively). Previous studies have "inferred that the receptor site(s) recognising esters as fruity-smelling probably possesses a nucleophilic amino acid residue for binding the carbonyl function, and hydrophobic regions for interacting with the alkyl side chains" (Squirrell, 1978).

Multiple receptors, which have now been found for several classes of pharmacologically active molecules (e.g. dopamine (Kebabian and Calne, 1979) and serotonin (Feroutka and Snyder, 1983)) may in fact be a general feature of vertebrate chemoreceptors (Shirley et al, 1984a).

Chemical modification studies on the olfactory mucosa of mammals must be carefully designed and interpreted because of the distinctive properties of the vertebrate olfactory system (see Polak, 1973; Poynder, 1974c; Beets, 1978; Dodd and Squirrell, 1980; Stoddart, 1980; Cagan and Kare, 1981): this system can respond to a wide range of stimuli including single odorants and complex mixtures.
The high discrimination of the olfactory system for such a large number of odours presents difficulties in formulating a model for the receptor process (see Polak, 1973; Dodd, 1974; Beets, 1979; Dodd and Persaud, 1981). A large number of specific receptor proteins seems unlikely from genetic and other standpoints. Conversely, it is not clear whether the differential reactions of odorants with the phospholipid regions of sensory membranes could provide sufficient discrimination in the primary neurone for the system to clearly identify odour type (Dodd and Persaud, 1981). Between these two contrasting hypotheses is the idea that there may be multiple receptor molecules for a particular odorant (Shirley et al, 1983a). The failure to abolish either the ECG responses to isovaleric acid with high concentrations of concanavalin A (Chapter 5) or the signals obtained to various heterocyclic odorants with appropriate vapour-phase labelling reagents (Chapter 3) may be interpreted as evidence for such receptor multiplicity (Shirley et al, 1986).

Support for this model has also been provided by various examinations of ligand binding. Studies on the interaction of $[^{3}H]2$-isobutyl-3-methoxypyrazine with olfactory tissue preparations have indicated the existence of four "binding species" (Felosi et al, 1981; see Section 6.4(ix); also see Felosi et al, 1980): the receptor from sheep which has been characterised in this thesis ($K_D = 1.68 \times 10^{-8} M$; see Section 6.4(vi)b) exhibited a much higher affinity for this odorant than that detected previously in homogenates of cow olfactory mucosa, which was saturable in the micromolar range ($K_D = 1.2 \mu M$; Felosi et al, 1982). The data obtained from other investigations (e.g. see Gennings et al, 1977; Cagan and Zeiger, 1978; Felosi et al, 1978; Fesenko et al, 1979; Fersaud, 1980) "suggest the
existence of two classes of binding sites in olfactory receptors" (Pelosi et al, 1982).

Several types of chemical-modification procedures have been shown to modify the response of olfactory mucosa (e.g. see Menevse et al, 1977b, 1978; Shirley et al, 1980, 1981; Criswell et al, 1980; Dodd and Persaud, 1981). Group-specific reagents would probably be regarded as the least likely to induce a specific effect since, from a mechanistic point of view, protein functionality is not known to be required at an odorant binding site. However, various group-specific reagents have modified the response of olfactory primary neurones, and differential effects have been achieved in protection experiments (see Getchell and Gesteland, 1972; Menevse, 1977; Menevse et al, 1978; Delaleu and Holley, 1980; Dodd and Persaud, 1981; Shirley et al, 1983a). In general, though, stronger and more specific effects may be observed with "affinity odorants" (e.g. see Squirrell, 1978, Dodd and Persaud, 1981) and these "may offer a more direct route to the identification of olfactory receptor proteins" (Shirley et al, 1983a); the covalent attachment of a radioactively-labelled ligand to molecular species at the active binding site of the receptor "will allow identification of the receptor macromolecules during further isolation and purification steps1" (Rhein and Cagan, 1981; also see Cagan, 1981). Additionally, the coupling of an affinity ligand to an insoluble column matrix (usually agarose) may permit the purification of receptor proteins by affinity chromatography; this technique was used in the attempted isolation of an anisole-binding protein from dog olfactory epithelium.

1 Markers such as carnosine (Margolis and Grillo, 1977; see Chapter 7) and the olfactory marker protein (Graziadei, 1971) are not specific to the sensory parts of the olfactory neurones, but are present throughout their length.

The reported isolation of a functionally active ciliary preparation from the olfactory rosettes of rainbow trout has supported the hypothesis that odorant recognition sites are integral parts of the cilia (Rhein and Cagan, 1980; see Section 1.3(1)); the detailed localisation of such sites along the ciliary membrane may be possible by employing a combination of biochemical approaches\(^1\) with electron microscopy. In addition, studies on the chemical composition of olfactory receptor membrane fractions from cilia may lead to a better understanding of the importance of membrane lipids in the initial events of olfactory sensation (Rhein and Cagan, 1981; also see Michell et al, 1976).

Most investigations into olfactory receptor mechanisms have, to date, employed the frog as an experimental animal for reasons of convenience (Gesteland and Sigwart, 1977; Shirley et al, 1983a). However, future studies may benefit from concentrating on one animal which can be investigated using behavioural, genetic and biochemical methods. The rat seems suitable for this purpose. Chemical modification studies performed on the frog \textit{in vivo} and \textit{in vitro} have been shown to give identical results (Squirrell, 1978), and so similar assumptions have been made about the rat preparation (Shirley et al, 1983a).

Future examinations of mammalian olfactory mechanisms should include further studies on the labelling of olfactory receptors, utilising both chemical modification and the antibody approach (Dodd

\(^{1}\) E.g. the covalent attachment of radiolabelled odorants (see Cagan, 1981) in possible combination with immunological approaches (see Goldstein and Cagan, 1981) "are exciting possibilities" (Rhein and Cagan, 1981).
and Persaud, 1981); investigations with a large number of odorant families may be necessary before the pattern of results obtained can be easily interpreted. The isolation of a pure preparation of olfactory cilia also represents an important line of investigation; a wide range of techniques are now available for characterisation of the tissue. At present, biochemical work on olfactory tissue homogenates is still in an early stage of development. Future work will need to correlate the results obtained from subcellular fractions with those of intact olfactory epithelium (Dodd and Persaud, 1981). The most potentially successful approach with humans appears to be the systematic synthesis and purification of the members of various odorant families, and the correlation of their olfactory properties with molecular structure (Dodd and Persaud, 1981). Although few systematic studies of this type have so far been performed, one such investigation with simple alkyl esters has succeeded in identifying the structural features responsible for their fruity-odour quality (Squirrell, 1978; also see Ohloff and Giersch, 1980; MacLeod, 1980; Ohloff et al., 1983).

Finally, it is interesting to compare the paucity of information presently available on the molecular mechanisms of olfaction with the progress recently made towards a fuller understanding of the visual system; in both, sensory reception takes place at ciliary organelles having large membrane area, and containing the molecular apparatus that mediates stimulus-evoked changes of membrane potential. Whilst the structure and function of rhodopsin (the photoreceptor protein of retinol rods) and some of its coupled enzymes are known in detail (e.g. see O'Brien, 1982; Zurer, 1983) "virtually no equivalent

1 For a review of current knowledge of the molecular transmitter systems inside the vertebrate rod outer segment, see Miller (1981).
information is available for their olfactory counterparts" (Chen and Lancet, 1984).

The visual response is initiated by light reception and transduction into chemical and electrical energy in the outer-segment membranes of rod and cone cells. Rods (see Figure 8.1) are elongated cells with a light-sensitive outer segment at one end and a synaptic body at the opposite terminus. Illumination of the outer segment alters the plasma membrane conductance causing hyperpolarisation of the cell membrane and subsequent modulation of synaptic transmitter release (see Hagins et al., 1970; Korenbrot and Cone, 1972). It is generally accepted that an internal transmitter is essential to communicate between light-sensitive rhodopsin molecules in the disc membranes of the rod outer segments (ROS) and the sodium ion permeability sites of the plasma membrane (Baylor and Fuortes, 1970). Two general approaches have advanced the understanding of vertebrate vision: electrophysiology has permitted delineation of the electrical response of intact photoreceptor cells, whilst chemistry has succeeded in isolating and characterising components of the ROS.

Current ideas (see Figure 8.2) suggest that light absorption by the chromophore (11-cis-retinal) of the disc transmembrane protein rhodopsin¹ (Figure 8.3) produces an intermediate (light-activated rhodopsin; Rh²) which catalyses the binding of GTP to a membrane surface-associated protein (G protein; see Baehr et al., 1982; also see Wheeler and Bitensky, 1977; Godchaux and Zimmerman, 1979; Robinson and Hagins, 1979; Fung and Stryer, 1980; Fung et al., 1981);

¹ Bovine rhodopsin is a single polypeptide with a molecular weight of about 38,000 (see Figure 8.3).
Figure 8.1 Schematic diagram of a vertebrate rod cell

Approximately $10^8$ rod cells are closely packed in a retina and are aligned with the incoming light.

Taken from O'Brien (1982)

Figure 8.2 An hypothesis for the activation sequence of cyclic GMP phosphodiesterase in rod outer segment membranes

\[
\begin{align*}
Rh \xrightarrow{hv} & \text{Rh}^* & \text{Light absorption} \\
G_{GDP} + GTP \xrightarrow{Rh^*} & G_{GTP}^* + GDP & \text{GTP binding} \\
PDE + G_{GTP} & \xrightarrow{PDE-G_{GTP}^*} \text{PDE activation} \\
\text{Cyclic GMP} + H_2O \xrightarrow{PDE-G_{GTP}^*} & \text{GMP} + H^+ & \text{Hydrolysis} \\
PDE - G_{GTP} \xrightarrow{GTP hydrolysis} & \text{PDE} + G_{GDP} & \text{Inactivation}
\end{align*}
\]

$\text{Rh}^*$: light-activated rhodopsin;

$G_{GDP}$: G protein with bound GDP;

$G_{GTP}^*$: G protein with bound GTP, the asterisk denoting an activated species;

PDE: phosphodiesterase

Taken from O'Brien (1982)
Figure 8.3  Schematic representation of rhodopsin and phospholipids in the disc membrane bilayer

Three of the $7 + 2$ α-helices of rhodopsin are shown; half-way down the first helix is the retinal binding site (lysine 53'). The 11-cis-retinylidene chromophore is parallel to the membrane surface and is presumed to lie between some of the α-helices.

Taken from O'Brien (1982)
as many as a few hundred GTP molecules are bound in exchange for GDP (one per G protein) for each Rh\(^\text{II}\) (Fung and Stryer, 1980). The light-activated G protein with bound GTP \((G\text{TP}\text{G})\) activates a cyclic nucleotide phosphodiesterase\(^1\) (Pannbacker et al., 1972; Miki et al., 1973) on the membrane surface by reducing inhibitory constraint (Hurley, 1980), which in turn catalyses the hydrolysis of cyclic GMP, thereby decreasing the cytoplasmic cyclic GMP concentration; about \(10^3\) molecules of cyclic GMP are hydrolysed per second. This reaction sequence may be modulated by the phosphorylation of Rh\(^\text{II}\) and it is terminated by the removal of the \(\gamma\)-phosphate of the bound GTP by guanosine triphosphatase activity\(^2\) (Wheeler and Bitensky, 1977; Wheeler et al., 1977; Robinson and Hagins, 1979) present in the ROS. The overall in vitro amplification is \(>10^5\) cyclic GMP molecules hydrolysed per second per Rh\(^\text{II}\). The cyclic nature of the relations is represented by the scheme shown in Figure 8.13.

The in vivo function of the light regulation of the cytoplasmic cyclic GMP concentration is unclear, although in vitro studies suggest that it precedes the light-induced change in receptor potential of the cell. Light absorption also increases the \(\text{Ca}^{2+}\)

---

\(^1\) Bovine phosphodiesterase is a holoenzyme which exhibits three subunits (at estimated molecular weights of 89,000, 84,000 and 13,000) upon sodium dodecyl sulphate discontinuous gel electrophoresis (Baehr et al., 1979); the preferred substrate is cyclic guanosine monophosphate (cyclic GMP) (Pannbacker et al., 1972; Miki et al., 1973) which is estimated to have a cytoplasmic concentration of 30 to 60\(\mu\)M (Woodruff et al., 1977; also see Yee and Liebman, 1978).

---

\(^2\) Electrophoresis of the material with GTPase activity shows three bands with estimated molecular weights of 37,000, 35,000 and 6,000 (Kuhn, 1980). In addition to displaying GTPase activity, these polypeptides, or the highest molecular weight (\(\alpha\)) unit alone (Fung et al., 1981), bind GTP upon light activation of the ROS (Godchaux and Zimmerman, 1979; Fung and Stryer, 1980).

---

\(^3\) The similarities between this sequence and the hormonally activated adenylate cyclase in the plasma membrane of other cells have been previously noted (Wheeler and Bitensky, 1977; Wheeler et al., 1977; Fung et al., 1981; Liebman and Pugh, 1980; also see Bitensky et al., 1982).
Figure 8.4. Cyclic scheme for the activation of cyclic GMP phosphodiesterase in rod outer segment membranes

\[
\begin{array}{c}
\text{Rh}^* \quad \text{GDP} \\
\text{GTP} \\
\text{Rh}^* \quad \text{G}_{\text{GTP}} \\
\text{G}_{\text{GDP}} \\
\text{Rh}^* \\
\text{G}_{\text{GTP}} \\
\text{G}_{\text{GDP}} \\
\text{PDE} \\
\text{H}_2\text{O} \\
\text{Pi} \\
\text{cyclicGMP} \\
\text{G}_{\text{GTP}} \\
\text{G}_{\text{GDP}} \\
\end{array}
\]

\[\text{Rh}^*: \quad \text{light-activated rhodopsin;}
\]

\[\text{G}_{\text{GDP}}: \quad \text{G protein with bound GDP;}
\]

\[\text{G}^*: \quad \text{G protein with bound GTP, the asterisk denoting an activated species;}
\]

\[\text{PDE}: \quad \text{phosphodiesterase}
\]

Taken from O'Brien (1982)
activity of the ROS cytoplasm; the relationship between the cyclic GMP chemistry and the cytoplasmic Ca$^{2+}$ activity is at present unresolved.

The role of Ca$^{2+}$ in ROS has been the subject of much investigation in recent years; several studies (e.g. see Bownds and Brodie, 1975; Lipton et al., 1977a,b; Brown et al., 1977; Szuts and Cone, 1977; Wormington and Cone, 1978; Woodruff and Bownds, 1979) have provided evidence in favour of this divalent ion functioning as an excitatory transmitter (Yoshikami and Hagins, 1971). The light-induced Ca$^{2+}$ release from the outer segments of photoreceptor cells in isolated retinas has been measured at >500 Ca$^{2+}$ per Rh, implying that light absorption by rhodopsin leads to an increase in the Ca$^{2+}$ activity of the ROS cytoplasm (Gold and Korenbrot, 1980; Yoshikami et al., 1980). The Ca$^{2+}$ release precedes the receptor potential, which implies that the increase in Ca$^{2+}$ activity regulates the plasma membrane sodium ion permeability (see Figure 8.5) (Gold and Korenbrot, 1980; Yoshikami et al., 1980). The hyperpolarisation of the cell modulates synaptic transmitter release and thereby controls cellular communication and information transmission.

The rapid progress of the past decade has been aided by the introduction of new techniques and the discovery of several ROS proteins, especially the peripheral enzymes. Conversely, however, the understanding of olfactory mechanisms "has advanced only a little beyond the situation outlined by Ohloff and Thomas (1971)" (Dodd and Persaud, 1981): despite an increased interest in the biochemical aspects of olfaction (see Poynder, 1974c; Benz, 1976), "in comparison with vision, there are few experimentalists" (Dodd and Persaud, 1981). It is conceivable though that the next few years may see an accelerated rate of progress: "we are in a period of emerging ideas..."
The plasma membrane of the rod cell outer segment has a high sodium ion permeability in the dark (left), which is reduced by light. The light-induced decrease in sodium ion permeability is accompanied by calcium ion fluxes from the interior of the outer segment to the interstitial space.

Taken from O'Brien (1982)
and hypotheses" (Mair and Gesteland, 1980). In particular, the recent studies on muscarinic receptor binding in the olfactory epithelium (e.g. see Hedlund et al, 1984; Section 6.1(iii)); electrophoretic mapping of membrane proteins in the cilia of frog olfactory epithelium (Chen and Lancet, 1984; see Section 5.4); cell-specific markers for the olfactory epithelium (e.g. see Hempstead and Morgan, 1983; Section 5.1(ii)), and nasal microsomal cytochrome P-450-dependent monooxygenases (e.g. see Dahl and Hadley, 1983; Section 1.3(ii)c) appear to offer much potential for future work. When considered alongside the concomitant increases in experimental sophistication and the number of personnel, it may be hoped that the rate of progress of the next decade mimics more closely that achieved over the past few years in understanding the visual process.
APPENDIX 1  FLAME IONISATION DETECTOR

The timing and the concentration of odorous stimuli applied to animal preparations, as well as the purity of the carrier gas, were monitored routinely by means of a flame ionisation detector adapted such that it continuously tested a small sample of the stream entering the nasal cavity (see Bostock and Poynder, 1972).

The sample of gas to be monitored was sucked directly into the detector (1-2 ml min\(^{-1}\)) through a 4 cm long probe of (0.64 mm o.d.) stainless tube (see Figure Al.1). The hydrogen for the flame was also fed into the open end of the probe (by means of a smaller (0.3 mm o.d.) tube hooked \(\frac{1}{2}\) mm into the inlet of the probe) to help sweep the sample rapidly towards the detector, thus minimising transfer and adsorption delays. Signals from the detector were amplified (Keithley 610B high impedance electrometer) and recorded on a Servoscribe chart recorder.

Since the inlet of the probe was so small, it was possible to sample discretely from points only 0.2 mm apart. The device itself has been used to check the switching efficiency of the odour applicator; to check that the applicator delivers a well mixed stream, and to determine accurately odorant concentrations: it has also been employed indirectly for measuring gas flow rates without disturbing them.
Figure A1.1  Device for monitoring a sequence of odour stimuli

F.I.D. is a flame ionisation detector; P is the probe; A is air (500 ml/min); H is hydrogen (50 ml/min); N is nitrogen (50 ml/min); E is exhaust connected to suction; S is sample stream (1 ml/min).
Inset shows enlargement of probe tip.

Taken from Bostock and Poynder (1972)
APPENDIX 2  The merits of employing frogs and rats as experimental animals in electrophysiological investigations

<table>
<thead>
<tr>
<th>FROGS</th>
<th>RATS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constitute an in vivo preparation</td>
<td>Constitute an in vitro preparation</td>
</tr>
<tr>
<td>Dissection under anaesthetic is slow and intricate</td>
<td>Dissection of severed head is quick and easy</td>
</tr>
<tr>
<td>Stable EOG responses often obtained only after considerable time ($\leq$1 hr.) because of the need to drain the olfactory eminence</td>
<td>Stable EOG responses usually obtained 15-20 min. after decapitation of the rat</td>
</tr>
<tr>
<td>Generally elicit smaller and slower EOG responses (rise time $\leq$2 sec.) than the rat</td>
<td>Generally elicit larger and faster EOG responses (rise time $\leq$1 sec.) than the frog</td>
</tr>
<tr>
<td>Preparation longevity ($\geq$5 hr.) is greater than the rat</td>
<td>Preparation longevity ($\leq$5 hr.) is less than the frog</td>
</tr>
<tr>
<td>Not readily amenable to other forms of olfactory investigation, thus precluding any link between biochemical, genetic and behavioural studies</td>
<td>Readily amenable to genetic and behavioural studies</td>
</tr>
</tbody>
</table>
APPENDIX 3 Determination of \( ^{3}H \)2-isobutyl-3-methoxypyrazine concentration and purity

The \( ^{3}H \)2-isobutyl-3-methoxypyrazine employed in the present ligand binding studies (see Chapter 6) was generously donated by Dr. P. Felosi, who has kindly supplied the following data relating to its concentration and purity.

A. Determination of the concentration of \( ^{3}H \)2-isobutyl-3-methoxypyrazine by ultraviolet absorption at 279 nm

(i) A calibration curve was constructed by preparing various dilutions of pure non-radioactive 2-isobutyl-3-methoxypyrazine (6.4 mg in 50 ml diethyl ether; \( 7.71 \times 10^{-4} \)M).

(ii) The concentration of the \( ^{3}H \)2-isobutyl-3-methoxypyrazine was determined by reference to this calibration curve.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>( A_{279} )</th>
<th>Concentration of sample</th>
<th>Concentration of undiluted solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 : 4</td>
<td>0.667</td>
<td>( 9.2 \times 10^{-5} )M</td>
<td>( 4.6 \times 10^{-4} )M</td>
</tr>
<tr>
<td>1 : 9</td>
<td>0.323</td>
<td>( 4.8 \times 10^{-5} )M</td>
<td>( 4.8 \times 10^{-4} )M</td>
</tr>
</tbody>
</table>

The mean value of the concentration of the \( ^{3}H \)2-isobutyl-3-methoxypyrazine = \( 4.7 \times 10^{-4} \)M.
B. Determination of the purity of $[3^\text{H}]2$-isobutyl-3-methoxy-pyrazine by thin-layer chromatography

(1) $[3^\text{H}]2$-Isobutyl-3-methoxy-pyrazine ($4.7 \times 10^{-4}$ M) in diethyl ether was spotted (~0.5 µl) onto a plastic-backed silica gel (0.2 mm thick) t.l.c. plate which was developed in benzene/chloroform/acetone (40 : 40 : 2). The plate was then cut into strips (6 mm) and analysed by liquid scintillation counting.

(2) As above, except that the t.l.c. plate was developed in chloroform only.
C. Determination of the purity of $[^3\text{H}]2$-isobutyl-3-methoxy-
aprazine by high-pressure liquid chromatography

The radiochemical purity of the $[^3\text{H}]2$-isobutyl-3-methoxy-
aprazine ($4.7 \times 10^{-4}\text{M}$ in 1,2-propanediol/water) was checked by HPLC using water/ethanol (60:40) as the eluent. The column eluate was fractionated (1 ml aliquots) and analysed by liquid scintillation counting.
APPENDIX 4: NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

All forms of absorption spectroscopy rely upon the fact that the absorption of radiation by a molecule involves an energy-level transition from its stable ground state to an excited state of higher energy. The occurrence of such a transition demands that the energy of the radiation quantum match the energy difference, $\Delta E$, between the ground and excited states, as specified by Planck's law,

$$\Delta E = \hbar \nu$$

where $\hbar \nu$ is the energy of the radiation quantum. Hence, a map of the energies of the excited states of the molecule is given by the spectrum of frequencies (or wavelengths) at which the molecule has strong absorptions; different molecular groups undergo absorptions at different parts of the spectrum (Knowles et al., 1976).

Nuclear magnetic resonance spectroscopy utilises electromagnetic radiation of radiofrequency, since the transition energies correspond to the weak interactions of a nucleus with a magnetic field; such interactions (i.e. the position at which the resonance occurs) are characteristic of the nature of the molecular structure and bonding, and the splittings in the spectrum are sensitively dependent on both molecular arrangement and bonding.

Magnetic resonance absorption can only be exhibited by species which possess a magnetic moment, since the prerequisite is that they should interact with a magnetic field in a similar fashion to a simple bar magnet; nuclei with an odd mass number possess such moments (see Table A4.1). The potential energy of a nucleus which has a net magnetic moment will be lower if the moment is aligned with the field than if it is pointing against it; magnetic resonance absorptions

\[\nu\] is the frequency of the radiation and $\hbar$ is Planck's constant.
<table>
<thead>
<tr>
<th>NMR</th>
<th>Abundance</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1$H, $^{13}$C</td>
<td>100%, 1%</td>
<td>All</td>
</tr>
<tr>
<td>$^{31}$P</td>
<td>100%</td>
<td>Nucleotides, phospholipids, phosphorylated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>metabolites</td>
</tr>
<tr>
<td>$^{14}$N, $^{15}$N, $^{33}$S</td>
<td>99.6%, 0.4%, 0.7%</td>
<td>Amino acids, peptides, proteins</td>
</tr>
<tr>
<td>$^{19}$F, $^3$H</td>
<td>100%, 0.2%</td>
<td>Isotopic label substituting for $^1$H</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ESR</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Transition metals</td>
<td></td>
<td>Metalloproteins, including metalloenzymes and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>haem proteins</td>
</tr>
<tr>
<td>Free radicals</td>
<td></td>
<td>Transient reaction intermediates, irradiated</td>
</tr>
<tr>
<td>Stable free radicals</td>
<td></td>
<td>species</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Spin labels: covalently attached or intercalated</td>
</tr>
</tbody>
</table>

Taken from Knowles et al (1976)
correspond to transitions between these energy levels\(^1\) (Figure A4.1). For the \(^1\)H nucleus, which possesses a spin of a half, only two orientations are permissible, depicted by the two outer spins of Figure A4.1 (known as the parallel and antiparallel orientations, or spin-up and spin-down). These two spin states are equally favourable in the absence of a magnetic field. However, the application of such a field results in the parallel orientation being preferred, since this set-up then has a lower energy. Transitions from the lower-energy spin orientation to the higher state can be induced by electromagnetic radiation, the position of the spectral absorption from a particular magnetic species being specified by the nuclear g-value (Figure A4.2\(^2\)).

The environment in which the magnetic nucleus resides influences not only the position of this absorption but also its appearance in the spectrum. The magnetic field experienced by a particular nucleus may be modified by a shielding effect of the surrounding electrons, the strength of the shielding depending upon the local electron bonding; this environmental factor is reflected by the line positions of a particular species being specified by a "chemical shift" relative to a standard compound (often TMS or DSS\(^3\)). The local field at any nucleus is also a function of the fields produced by other magnetic nuclei in its vicinity. The interaction of the central magnetic moment with the magnetic moments of the

---

1 Nuclear spins are quantized such that they can only take up certain orientations with respect to the magnetic field, each corresponding to a distinct energy.

2 Magnetic resonance spectroscopy differs from many other forms of spectroscopy in that, for technical reasons, scanning a spectrum usually involves sweeping the magnetic field rather than the frequency.

3 Tetramethyl silane (TMS); 2,2-dimethylsilapentane-5-sulphonic acid (DSS).
Figure A4.1  Magnetic moment, $\mu$, in minimum, intermediate and maximum (left to right) energy orientations with respect to a magnetic field $H$

Taken from Knowles et al (1976)

Figure A4.2  Energy-level splitting and magnetic resonance transition for an electron or nucleus, of a spin of one-half, in a magnetic field

$\mu$, $\Sigma$

-1/2, +1/2

$\nu = \beta H$

(\( \beta \) = Bohr magneton; 9.27 x 10^{-24} JT$^{-1}$)

+1/2, -1/2

Taken from Knowles et al (1976)
surrounding nuclei gives rise to spin-spin splittings in the NMR spectrum, the magnitude of such splitting being reflected by the "coupling constant" \( (J) \) for the interaction. In general, if the neighbouring group contains \( n \) hydrogen nuclei, then the spectral line under consideration will be split into \((n+1)\) components. Clearly, the complexity of the splitting pattern will be influenced by the number of nuclei involved; such splitting is characteristic of the molecular arrangement experienced by a particular nucleus. \(^1\)

Figure A4.2 has depicted resonance absorption as a spin transition from the low-energy parallel orientation to the higher-energy antiparallel orientation. However, spin flip transitions in the opposite direction with concomitant emission of radiation of the same frequency, are just as effectively caused by electromagnetic radiation; a net absorption of radiation will accrue only if initially fewer spins exist in the upper rather than the lower state. Although this is always the case when the spins are in thermal equilibrium (since it is energetically more favourable for a spin to be in the lower energy level), this equilibrium does not necessarily apply to a system undergoing electromagnetic transitions; the populations in the upper and lower levels will quickly become equalized, unless the excess spins in the upper state are able to externally dissipate energy and thereby maintain the thermal equilibrium Boltzmann distribution by dropping down into the lower state. This process of losing energy externally is known as spin-lattice relaxation (longitudinal relaxation) and the speed with which it occurs is characterised by the spin-lattice relaxation time, \( T_1 \). This relaxation time is dependent upon the molecular environment (i.e. how strongly the spin is "coupled"

\(^1\) For a more detailed account of line multiplicity in NMR, see Knowles et al (1976).
to the lattice), and is short in solids but relatively long in liquids (slow relaxation). The effect of this may be evidenced in the occurrence of line broadening or signal saturation, although the disappearance of lines due to relaxation time broadening is only encountered in certain systems (e.g. strongly paramagnetic; containing quadrupolar nuclei such as $^{14}$N or $^{35}$Cl).

The magnetic interaction between nuclei and paramagnetic electrons (the magnetic dipole-dipole interaction) is an extremely efficient means of relaxing nuclei from their upper spin state. However, it is possible that magnetic interactions may also occur between the nuclei themselves. One of the effects of the dipolar interaction between like spins is to cause mutual spin flips. Unlike spin-lattice relaxation, this spin-spin relaxation (transverse relaxation) does not alleviate saturation but contributes to the lifetime broadening of the line. Under normal circumstances, it is the spin-spin relaxation time, $T_2$, and not $T_1$ which determines the intrinsic linewidth.

The rate at which spin exchanges take place, and hence the relaxation time $T_2$, depends upon the extent to which nuclei are exposed to each other's varying fields. For structures in which the relative motion of the nuclei is slow (i.e. rigid structures), the process is quite efficient such that $T_2$ is correspondingly short; with increasing molecular motion, smaller molecular weights, higher temperatures or more flexible molecules, this efficiency diminishes. Convenience demands that a quantitative expression be applicable to rates of motion of molecules relative to each other; this is achieved by means of a "correlation time" ($\tau_0$), which may be generally defined as the time taken by a molecule to turn through one radian or move through one molecular distance.
These considerations indicate that the relaxation times of a given group of nuclei, as reflected by the linewidth of its resonance, may be used to indicate its rate of motion in solution. Biologically, this is very important, since the rate of motion of a given group of nuclei may be considerably altered if the molecule or part of a molecule containing it undergoes transition from a flexible to a rigid form or vice versa; aggregates with other molecules; or interacts with larger or more rigid molecules.
APPENDIX 5

acetic acid  
acetophenone  
2-acetyl-3-methylpyrazine  
2-acetylpyrazine  
2-, 3-acetylpyridine  
aCRYlC acid  
t-butanethiol  
4-buTy1-5-propylthiazole  
n-butyric acid  
cAMPHOR  
(-) carvone  
(+) carvone  
1,8-ciNeole  
citronellol  
m-cresol  
cyCLOdOdECYL propionate  
cyCLOpентаCNeCARBOXylic acid  
deCanal  
diACEtvl  
DiMeTHyLEThyLPyrazine  
3,5-diTERtyRiarybutylacetophenone  
ethyL 2-pyrazinecarboxylate  
isCamyl acetate  
2-ISOBuTyL-3-mETOxyPyrazine  
2-ISOpropyl-3-mETOxyPyrazine  
isovaleriiC acid  
Mechthone  

ODOUR DESCRIPTIONS

strong, pungent, characteristic  
pungent, sweet  
cereal, roast grain  
popcorn  
tobacco-like  
strong, pungent, characteristic  
characteristic, putrid  
green, bell pepper  
persistent, penetrating, rancid, butter-like  
characteristic, penetrating  
spearmint  
caraway  
camphoraceous  
floral, rose  
phenolic  
woody  
phenolic, ethereal, sweaty, unpleasant  
pronounced, fatty; floral on dilution  
penetrating; butter-like on dilution  
smoked, burnt  
sweet, warm  
sweet, fruity  
powerful, fruity  
green, bell pepper  
earty, raw potato, bell pepper  
unpleasant, rancid, sweaty  
characteristic; similar to menthol
2-methylpyrazine: strong; chocolatey on dilution
2-methyl-3-methoxypyrazine: nutty, earthy, roasted peanuts
nicotinic acid: weak, sharp
n-octanethiol: penetrating, characteristic, putrid alcoholic
n-propanol: powerful, warm, orange blossom-like alcoholic
propiophenone: weak, ethereal
2-pyrazinecarboxylic acid: penetrating, warm, characteristic
2-, 3-pyridinemethanol propionate: warm, unpleasant, characteristic
3-pyridylacetic acid: warm, characteristic
3-pyridylacetonitrile: strong, penetrating, fishy
trimethylamine: baked potato, roasted peanut
trimethylpyrazine:

These descriptions are either taken from Arctander (1969), Amoore (1970a), Kaga and Sizer (1975), or are based upon quality judgements made by a panel of three people (including Dr. G.H. Dodd, a trained perfumer) on three separate occasions.
REFERENCES


Allison, A.C. (1953) 'The morphology of the olfactory system in vertebrates' Biol. Rev. 28, 159-244

Allison, A.C. and Warwick, R.T.T. (1949) 'Quantitative observations on the olfactory system of the rabbit' Brain 72, 156-197


Amoore, J.E. (1952) 'The stereochemical specificities of human olfactory receptors' Perf. and Essent. Oil Record 43, 321-330


Amore, J.E. (1967) 'Specific anosmia: a clue to the olfactory code' Nature (London) 214, 1095-1098


Amoore, J.E., Palmieri, G., Wanke, E. and Blum, M.S. (1969) 'Ant alarm pheromone activity: correlation with molecular shape by scanning computer' Science 165, 1256-1259

Amoore, J.E., Pelosi, P. and Forrester, L.J. (1977) 'Specific anosmias to 5α-androst-16-en-3-one and w-pentadecalactone: the urinous and musky primary odors' Chem. Sens. Flavor 2, 401-425


Arctander, S. (1969) 'Perfume and Flavour Chemicals' Arctander, Montclair, New Jersey


Baehr, W., Devlin, M.J. and Applebury, M.L. (1979) 'Isolation and characterization of cGMP phosphodiesterase from bovine rod outer segments' J. Biol. Chem. 254, 11669-11677


Barber, R., Primrose, S. and Dodd, G.H. (1979) 'Photoaffinity labelling of a chemoreceptor protein in Pseudomonas' FEBS Letts 105, 43-46


Blaha, G., Blair, W., Nickell, W.T. and Shipley, M.T. (1984) 'Nicotinic (N) and Muscarinic (M) Cholinergic receptors are segregated and coincide with acetylcholinesterase (ACHE) localization in rat olfactory bulb' Abstr. 21 The Sixth Annual Meeting of the Association for Chemoreception Sciences, Sarasota, Florida


Boelens, H. (1974)  'Relationship between the chemical structure of compounds and their olfactive properties' Cosmet. Perfum. 69, 1-7


Bostock, H. and Poynder, T.M. (1972)  'Apparatus for delivering and monitoring a sequence of odour stimuli' J. Physiol. 224, 14P-15P


Bronstein, A. A. and Minor, A. V. (1977) 'The regeneration of olfactory flagella and restoration of the electroolfactogram after treatment of the olfactory mucosa with Triton X-100' (In Russ.) Tsitologiya 12, 33-38


<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown, J.E., Coles, J.A. and Pinto, L.H.</td>
<td>(1977) 'Effects of injections of calcium and EGTA into the outer segments of retinal rods of Bufo marinus' J. Physiol. 262, 707-722</td>
</tr>
</tbody>
</table>


Cain, W.S. (1976) 'Olfaction and the common chemical sense: some psychophysical contrasts' Sensory Processes 1, 57-67


Calabretta, P.J. (1978) 'Synthesis of some substituted pyrazines and their olfactory properties' Perfumer & Flavorist 2, 33-42


Chow, B.F. and McKee, C.M. (1945) 'Interaction between crystalline penicillin and human plasma proteins'
Science 101, 67-68


Constanzo, R.M. and O'Connell, R.J. (1978) 'Spatially organised projection of hamster olfactory nerves' Brain Res. 139, 327-332

Cowper, R.K. and Davidson, L.H. (1939) 'w-Bromoacetophenone'
Org. Syntheses 19, 24-26

Science 210, 425-426


Cuatrecasas, P. and Hollenberg, M.D. (1976) 'Membrane receptors and hormone action'
Adv. Protein Chem. 20, 251-451

Cuatrecasas, P., Fuchs, S. and Anfinsen, C.B. (1967) 'The binding of nucleotides and calcium to the extracellular nuclease of Staphylococcus aureus; studies by gel filtration'
J. Biol. Chem. 242, 3063-3067


Davies, J. T. (1953) 'L'odeur et la morphologie des molecules' Industr. Parfum. 8, 74-79


Davies, J. T. (1969) 'The "penetration and puncturing" theory of odor: types and intensities of odors' J. Colloid and Interface Science; Schulman Memorial Volume 22, 296-304


Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1972)  
Eds: 'Data for Biochemical Research'  

Deck, R.E. and Chang, S.S. (1965)  
'Identification of 2,5-dimethylpyrazine in the volatile flavor compounds of potato chips'  
Chem. Ind. (London) 1343-1344

Modification of transduction mechanisms in the frog's olfactory mucosa using a thiol reagent as olfactory stimulus'  
Chem. Senses Flavour 2, 205-218

Delaleu, J.C. and Holley, A. (1983)  
'Investigations of the discriminative properties of the frog's olfactory mucosa using a photoactivable odorant'  
Neuroscience Letters 17, 251-256

De Lorenzo, A.J.D. (1963)  
'Studies on the ultrastructure and histophysiology of cell membranes, nerve fibers and synaptic junctions in chemoreceptors' in: 'Olfaction and Taste I'  
Ed. by Zotternan, Y. Pergamon Press, Oxford pp 5-17


'Thiamine-dependent accumulation of tetramethylpyrazine accompanying a mutation in the isoleucine-valine pathway'  
J. Bacteriol. 94, 323-326

Demerdache, A. and Wright, R.H. (1967)  

'Purinergic olfactory receptors: Electrophysiological and behavioural evidence'  
Abstr. 38 The Sixth Annual Meeting of the Association for Chemoreception Sciences, Sarasota, Florida

De Reuck, A.V.S. and Knight, J. (1968)  
Eds: 'Hearing mechanisms in vertebrates' (A Ciba Foundation Symposium)  

DeSimone, J.A. (1981)  
'Physicochemical principles in taste and olfaction' in: 'Biochemistry of Taste and Olfaction' Ed. by Cagan, R.H. and Kare, M.R.  
DeSimone, J.A. (1981) 'Hydrodynamic and surface chemical effects of chemo- sensory stimuli' Abstr. 6 The Sixth Annual Meeting of the Association for Chemoreception Sciences, Sarasota, Florida


Dodd, G.H. (1978) 'Biochemical aspects of coding and transduction in the primary olfactory neurones' Drug Res. 28, 2362


Dodd, G.H., Barratt, M.D. and Rayner, L. (1970) 'Spin probes for binding site polarity' FEBS Letts. 8, 286-288


Döving, K.B. (1964) 'Studies of the relation between the frog's electro-olfactogram (EOG) and single unit activity in the olfactory bulb' Acta Physiol. Scand. 66, 150-163


Duncan, C.J. (1964) 'The transducer mechanism of sense organs' Naturwissenschaften 51, 172-173


Identification of neutral and anionic 8α-substituted flavin semiquinones in flavoproteins by electron spin resonance spectroscopy' Arch. Biochem. Biophys. 208, 69-74


Eyzaguirre, C. and Kuffler, S.W. (1955)

Processes of excitation in the dendrites and in the soma of single isolated sensory nerve cells of the lobster and crayfish' J. Gen. Physiol. 39, 87-119


'The use of NBD-chloride (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) in detecting amino acids and as an N-terminal reagent' Anal. Biochem. 51, 290-294

Farquhar, M.G. and Palade, G.E. (1963)


Farquhar, M.G. and Palade, G.E. (1965)


'Microperoxidase, a new tracer of low molecular weight. A study of the interstitial compartments of the mouse brain' J. Cell Biol. 43, 35a-36a


'Basis issues in binding' Trends Biochem. Sci. 6(7), IV-VII

Ferriero, D. and Margolis, F.L. (1975)

'Denervation in the primary olfactory pathway of mice. II. Effects on carnosine and other amine compounds' Brain Res. 24, 75-86

Ferron, J. (1973)

'Morphologie comparee de l'organe de l'odorat chez quelques mammiferes carnivores' Naturaliste Canad. 100, 525-541


'Molecular mechanisms of olfactory reception. IV. Some biochemical characteristics of the camphor receptor from rat olfactory epithelium' Biochim. Biophys. Acta 587, 424-433


Frazier, J.L. and Heitz, J.R. (1975) 'Inhibition of olfaction in the moth Heliothis virescens by the sulphydryl reagent fluorescein mercuric acetate' Chem. Senses Flavor 1, 271-281.


Gesteland, R.C. (1984) 'On knowing stimulus intensity at the receptor' Abstr. 8 The Sixth Annual Meeting of the Association for Chemoreception Sciences, Sarasota, Florida


Gesteland, R.C., Lettvin, J.Y. and Pitts, W.H. (1965) 'Chemical transmission in the nose of the frog' J. Physiol. 181, 525-559


Getchell, T. V. and Shepherd, G. M. (1978) 'Responses of olfactory receptor cells to step pulses of odour at different concentrations in the salamander'. J. Physiol. 282, 521-540


Godchaux, W. and Zimmerman, W. F. (1979) 'Membrane-dependent guanine nucleotide binding and GTPase activities of soluble protein from bovine rod cell outer segments'. J. Biol. Chem. 254, 7874-7884


Gonzalez, M.T. and Freeman, W.J. (1979) 'Hydrogen ion buffers for biological research' Biochemistry 5, 467-477


Graziadei, P.P.C. (1971) 'The olfactory sensory neuron as a model for the study of regeneration' Abstracts 3rd ECRO Congress, Pavia p 60

Graziadei, P.P.C. and DeHan, R.S. (1973) 'Neuronal regeneration in frog olfactory system' J. Cell Biol. 52, 525-530


"Olfactory studies on enantiomeric eremophilane sesquiterpenoids"  
J. Agric. Food Chem. 20, 1018-1021

Hartman, F.C. (1977)  
"Haloketones as affinity labeling reagents"  
in 'Affinity Labeling' Ed. by Jakoby, W.B. and Wilchek, M.  
'Methods in Enzymology' (Eds.: Colowick, S.P. and Kaplan, N.O.)  

Hayward, L.D. (1977)  
'A new theory of olfaction based on dispersion induced optical activity'  
Nature (London) 267, 554-555

Hedlund, B. and Bartfai, T. (1979)  
"The importance of thiol- and disulfide groups in agonist and antagonist binding to the muscarinic receptor"  
Mol. Pharmacol. 15, 531-544

Hedlund, B. and Shepherd, G.M. (1983)  
"Biochemical studies on muscarinic receptors in the salamander olfactory epithelium"  
FEBS Letts. 162, 428-431

"Vasoactive intestinal polypeptide and muscarinic receptors: supersensitivity induced by long-term atropine treatment"  
Science 220, 519-521

"Is the olfactory receptor related to the muscarinic cholinergic receptor?"  
(personal communication)

Heist, H.E., Mulvaney, B.D. and Landis, D.J. (1967)  
'Odour sensing cells: ultra-structure by electron microscopy'  
Honeywell Inc. Corporate Res. Centre, Hopkins, Minnesota, U.S.A.

Hempstead, J.L. and Morgan, J.I. (1983)  
'Fluorescent lectins as cell-specific markers for the rat olfactory epithelium'  
Chemical Senses 8, 107-120

'Specific anosmia to exaltolide: selection criteria' in 'Olfaction and Taste VII'  
Ed. by van der Starre, H. IRL Press Ltd., London and Washington DC p 431

Hendrickson, J.B., Cran, D.J. and Hammond, G.S. (1970)  

Higashino, S. and Takagi, S.F. (1964)  
'The effect of electrotonus on the olfactory epithelium'  
J. Gen. Physiol. 48, 325-335
Hirsch, J.D. and Margolis, F.L. (1979)  
"L-[³H]-carnosine binding in the olfactory bulb. II. Biochemical and biological studies" Brain Res. 174, 81-94


'Ligand binding studies in the mouse olfactory bulb: identification and characterization of a L-[³H]-carnosine binding site' Brain Res. 158, 407-422

'The effect of sodium ions on the electrical activity of the giant axon of the squid' J. Physiol. (Lond.) 108, 37-77

Holden, A.V. (1976)  


Holley, A. (1974)  
'The discriminating power of the vertebrate olfactory receptors' in 'Transduction mechanisms in chemoreception' Ed. by Foynder, T.M. IRL Press Ltd., London. pp 275-289

Holley, A. and Davring, K.B. (1977)  
'Receptor sensitivity, acceptor distribution, convergence and neural coding in the olfactory system' in 'Olfaction and Taste VI' Ed. by Le Lagnen, J. and MacLeod, F. IRL Press Ltd., London. pp 113-123

'Qualitative and quantitative discrimination in the frog olfactory receptors: analysis from electrophysiological data' in 'Odors: Evaluation, Utilization, and Control' Annals of the New York Academy of Sciences Vol 237 (Ed. by Cain, W.S.) pp 102-114

Hopkins, A.E. (1926)  
'The olfactory receptors in vertebrates' J. Comp. Neurol. 41, 253-269

'Purification and characterization of carnosine synthetase from mouse olfactory bulbs' J. Neurochem. 31, 909-914
<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Title</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jackson, R.T. and Lee, C.C. (1965)</td>
<td>Degeneration of olfactory receptors induced by colchicine</td>
<td>Experimental Neurol. 11, 483-492</td>
</tr>
</tbody>
</table>

Jardetzky, O., Fischer, J.J. and Pappas, P. (1961) 'Nuclear magnetic resonance studies of the binding sites of biologically active substances. I. Method and protein binding' Biochem. Pharmacol. 8, No. 1, Abstr. 387


Kanda, T., Kitamura, T., Kaneko, T. and Tizumi, O. (1973) 'Scanning electron microscopical observations on the olfactory epithelium of guinea pig and human being' J. Otolaryngol. (Japan) 76, 30 (cited by Menco, 1977a)


Kumamoto Med. J. 14, 37-46

Kistiakowski, G.B. (1950) 'On the theory of odors'
Science 112, 154-155


J. Neurochem. 16, 145-157

Koch, R.B. (1971/1972) 'Responses of ATPase activities to octanol and ascorbic acid'


Koch, R.B. and Lesaiah, D. (1974) 'Preliminary studies on rat olfactory tissue: effects of odorants on Na\(^{+}\)-K\(^{+}\)-ATPase activity'
Life Sci. 15, 1005-1015

Koch, R.B. and Gilliland, T.I. (1977) 'Responses of Na\(^{+}\)-K\(^{+}\)-ATPase activity from dog olfactory tissue to selected odorants'
Life Sci. 20, 1051-1062

Koch, R.B., Rossi, H. and Price, S. (1980) 'Effect of antibody against anisole binding protein on odorant perturbation of Na\(^{+}\)-K\(^{+}\)-ATPase activity'
The Second Annual Meeting of the Association for Chemoreception Sciences, Sarasota, Florida.

Chem. Sens. 6, 95-99

J. Agric. Food Chem. 17, 393-396

Koehler, P.E., Mason, M.E. and Odell, G.V. (1971) 'Odor threshold levels of pyrazine compounds and assessment of their role in the flavor of roasted foods'
J. Food Sci. 36, 816-818

Koehler, P.E. and Odell, G.V. (1970) 'Factors affecting formation of pyrazine compounds in sugar-amine reactions'
J. Agric. Food Chem. 18, 895-898
Korenbrot, J.I. and Cone, R.A. (1972)  
'Dark ionic flux and the effects of light in isolated rod outer segments'  
J. Gen. Physiol. 60, 20-45

Korman, S. and Clarke, H.T. (1956)  
'Carboxymethyl amino acids and peptides'  
J. Biol. Chem. 221, 113-131

'Discovery of a pyrazine in a natural product: tetramethylpyrazine from cultures of a strain of Bacillus subtilis'  
Nature (London) 193, 776

Koyama, N. and Kurihara, K. (1972)  
'Effect of odorants on lipid monolayers from bovine olfactory epithelium'  
Nature (London) 236, 402-404

'Isolation and some properties of plasma membranes from bovine olfactory epithelium'  
Biochim. Biophys. Acta 241, 42-48

Kramer, G. (1933)  
'Untersuchungen über die sinnesleistungen und das orientierungsverhalten von Xenopus laevis, DAUD'  
Zool. Jahrb. Physiol. 52, 629-676

Kratzing, J. (1978)  
'The olfactory apparatus of the bandicoot (Isoodon macrourus): fine structure and presence of a septal olfactory organ'  
J. Anat. 125, 601-613

'Inherent spatial patterning of responses to odorants in the salamander olfactory epithelium' in 'Olfaction and Taste VII'  

Kuhn, H. (1980)  
'Light- and GTP-regulated interaction of GTPase and other proteins with bovine photoreceptor membranes'  
Nature (London) 283, 587-589

Kurihara, K. (1967)  
'Isolation of chromoproteins from bovine olfactory tissues'  
Biochim. Biophys. Acta 148, 328-334

Kurihara, K. and Koyama, N. (1972)  
'High activity of adenyI cyclase in olfactory and gustatory organs'  
Biochem. Biophys. Res. Commun. 48, 30-34

'Molecular mechanisms of transduction in chemoreception' in 'Biochemistry of Taste and Olfaction'  
Laffort, P. (1963) 'Essai de standardization des sevils olfactifs humains pour 192 corps purs' Arch. Sci. Physiol. 17, 75-105


Laurent, A. (1844) Ann. 52, 356-357


'ATP mediates rapid reversal of cyclic GMP phosphodiesterase activation in visual receptor membranes'
Nature (London) 287, 734-736

'Analyzing nonlinear Scatchard plots'
Science 223, 76-77

'Electrical and adaptive properties of rod photoreceptors in Bufo marinus. I. Effects of altered extracellular calcium ion levels' J. Gen Physiol. 70, 747-770

'Electrical and adaptive properties of rod photoreceptors in Bufo marinus. II. Effects of cyclic nucleotides and prostaglandins' J. Gen. Physiol. 70, 771-791

'Fine structure of the olfactory epithelium in some primates' J. Anat. 123, 135-145

'Protein measurement with the Folin phenol reagent' J. Biol. Chem. 193, 265-275

'On the nature of the universe'

'Vasoactive intestinal polypeptide enhances muscarinic ligand binding in cat submandibular salivary gland'
Nature (London) 295, 117-119

'Topographic coding of olfactory quality: odorant-specific patterns of epithelial responsivity in the salamander'
J. Neurophysiol. 48, 584-596


'Premieres donnes sur l'electro-olfactogramme du lapin' J. Physiol. (Paris) 51, 85-92

'Responses of olfactory bulb neurones to the dipeptide carnosine'
Exp. Brain Res. 34, 163-168
Maga, J.A. and Sizer, C.S. (1973)
'Myrazines in foods. A review'
J. Agric. Food Chem. 21, 22-30

Maga, J.A. and Sizer, C.S. (1975)
'Myrazines in foods' in 'Fenaroli's Handbook of Flavor Ingredients' (2nd edition)


'Carnosine in the primary olfactory pathway' Science 184, 909-911

Margolis, F.L. (1975)


Margolis, F.L. and Grillo, M. (1977)
'Axoplasmic transport of carnosine (ß-alanyl-L-histidine) in the mouse olfactory pathway' Neurochem. Res. 2, 507-519

'Enzymatic and immunological evidence for two forms of carnosinase in the mouse' Biochim. Biophys. Acta 570, 311-323


'Immunosuppressive activity of concanavalin A' Science 163, 476

'Fast and loose covalent binding of ketones as a molecular mechanism in vertebrate olfactory receptors. Chemical production of selective anosmia' Tetrahedron 40, 483-492

Mathews, D.F. (1972) 'Response patterns of single neurones in the tortoise olfactory epithelium and olfactory bulb' J. Gen. Physiol. 60, 166-180


Matthes, E. (1927) Z. Verg. Physiol. 5, 83 (cited by Otteson, 1956)


Menco, B.P.M. (1977b) 'Freeze-etch morphology of olfactory and respiratory cilia in rat, cow and frog' in 'Olfaction and Taste VI' Ed. by Le Wagenen, J. and MacLeod, P. IRL Press Ltd., London. p 199

Menco, B.P.M. (1978) 'Freeze-fracture studies on olfactory and respiratory cilia and microvilli in frog, rat and dog' Abstract: 3rd ECN0 Congress, Pavia.

Menco, B.P.M. (1980a) 'Qualitative and quantitative freeze-fracture studies on olfactory and nasal respiratory structures of frog, ox, rat, and dog. I. A general survey' Cell Tissue Res. 207, 183-209

Menco, B.P.M. (1980b) 'Qualitative and quantitative freeze-fracture studies on olfactory and nasal respiratory epithelial surfaces of frog, ox, rat, and dog. II. Cell apices, cilia, and microvilli' Cell Tissue Res. 211, 5-29
Menco, B.P.M. (1980c) 'Qualitative and quantitative freeze-fracture studies on olfactory and nasal respiratory epithelial surfaces of frog, ox, rat, and dog. III. Tight-junctions' Cell Tissue Res. 211, 361-375

Menco, B.P.M. (1980d) 'Qualitative and quantitative freeze-fracture studies on olfactory and respiratory epithelial surfaces of frog, ox, rat, and dog. IV. Ciliogenesis and ciliary necklaces (including high-voltage observations)' Cell Tissue Res. 212, 1-16


Metzger, H., Wofsy, L. and Singer, S.J. (1963)

'Receptor occupancy dose-response curve suggests that phosphatidylinositol breakdown may be intrinsic to the mechanism of the muscarinic cholinergic receptor' FEBS Letts. 69, 1-5


'Regulation of cyclic nucleotide concentrations in photoreceptors: an ATP-dependent stimulation of cyclic nucleotide phosphodiesterase by light' Proc. Natl. Acad. Sci. (U.S.A.) 70, 3820-3824

Mildvan, A.S. and Engle, J.L. (1972)


Miles, W.R. and Beck, L.H. (1949)


Minor, A.V. and Sakina, N.L. (1973)

'The role of cAMP in olfactory reception' (in Russ.) Neurofiziologiya 5, 414-422

Moncrieff, R.W. (1949)

'What is odor? A new theory' Am. Perfumer Essent. Oil Rev. 54, 453-454

Moncrieff, R.W. (1951)


Moncrieff, R.W. (1954)

'The odorants. Basic odor research correlation' Annals of the New York Academy of Sciences 50, 75-82

Moncrieff, R.W. (1955)

'The sorptive properties of the olfactory membrane' J. Physiol. (London) 130, 543-558

Moncrieff, R.W. (1967)


Moulton, D.G. (1976) 'Spatial patterning of response to odours in the peripheral olfactory system' Physiol. Rev. 56, 578-593

Moulton, D.G. and Beidler, L.M. (1967) 'Structure and function in the peripheral olfactory system' Physiol. Rev. 47, 1-52


Mozell, M. M. (1964b) 'Olfactory discrimination: electrophysiological spatiotemporal basis' Science 143, 1336-1337

Mozell, M. M. (1966) 'The spatiotemporal analysis of odorants at the level of the olfactory receptor sheet' J. Gen. Physiol. 50, 25-41


Muller, A. (1955) 'Quantitative untersuchungen am reichepithel des hundes' Z. Zellforsch 41, 335-350


Mustaparta, H. (1971) 'Spatial distribution of receptor responses to stimulation with different odours' Acta Physiol. Scand. 82, 154-166


Ng, R. H. and Marshall, F. D. (1976) 'Regional and subcellular distribution of homocarnosine - carnosine synthetase in the central nervous system' J. Neurochem. 30, 187-190
Ng, R.H., Marshall, F.D., Henn, F.A. and Sellstrom, A. (1977) 
"Metabolism of carnosine and homocarnosine in subcellular fractions and neuronal and glial cell-enriched fractions of rabbit brain" J. Neurochem. 28, 449-452

"Lymphocyte transformation induced by concanavalin A and its reversion by methyl-(x-D-mannopyranoside" Biochim. Biophys. Acta 228, 579-583

"Molecular mechanisms of odor sensing. V. Some biochemical characteristics of the alanineous receptor from the olfactory epithelium of the skate Dasynatis pastinaca" Chemical Senses 2, 195-203

"The chemistry of vision" Science 218, 961-966

"Quantitative stimulation of frog olfactory receptors" J. Neurophysiol. 32, 51-63

Ogle, W. (1870) 
"Anosmia (or cases illustrating the physiology and pathology of the sense of smell)" Med.-Chir. Trans. 55, 263-290

Chloff, G. (1972) 
"Odorous properties of enantiomeric compounds" in 'Olfaction and Taste IV' Ed. by Schneider, D. Wiss. Verlag, K.B.H., Stuttgart. pp 156-160

Chloff, G. (1980) 

Chloff, G. and Flament, I. (1979) 
"The role of heteroatomic substances in the aroma compounds of foodstuffs' Fortschr. Chem. Org. Naturst. 26, 231-283


Chloff, G. and Thomas, A.K. (1971) 
Eds.: 'Gustation and Olfaction' Academic Press, New York


Ottoson, D. (1959b)  "Comparison of slow potentials evoked in the frog's nasal mucosa and olfactory bulb by natural stimulation" Acta Physiol. Scand. 47, 149-159


Pelosi, P. and Pisanelli, A.M. (1980) 'Specific anosmia to 1,6-cineole: the camphor primary odour' Chem. Senses 6, 87-93


Abstract: 3rd ECRO Congress, Pavia, Italy.


Flattig, K.H. and Kobal, G. (1978)  
'Human EOG and olfactory evoked brain potentials: techniques and reliability' Results presented in a lecture given at the 3rd ECHO Congress, Pavia.

Pober, J.S. and Bitensky, M.W. (1979)  
'Light regulated enzymes of vertebrate retinal rods' Adv. Cyclic Nucleotide Res. 11, 265-301

Polak, E.H. (1975)  
'Multiple profile - multiple receptor site model for vertebrate olfaction' J. Theor. Biol. 40, 469-484

Polak, E. (1983)  
'Is odour similarity quantifiable?' Chem. Ind. (London) pp 30-36

'Effect of concanavalin A on rat olfactory mucosa' Abstract: 5th ECHO Congress, Regensburg.

'Reversible interaction of human lymphocytes with the mitogen concanavalin A' Exptl. Cell Res. 62, 315-325

Poynder, T.M. (1974a)  
'Response of the frog olfactory system to controlled odour stimuli' J. Soc. Cosmetic Chem. 25, 183-202

Poynder, T.M. (1974b)  

Poynder, T.M. (1974c)  

'Recording of electrical activity from mammalian olfactory epithelium in vitro: stimulation with pheromones and other odorants' Abstract: 3rd ECHO Congress, Pavia.

Price, S. (1977)  
'Specific anosmia to geraniol in mice' Neurosci. Lett. 4, 49-50

Price, S. (1978)  
'Anisole binding protein from dog olfactory epithelium' Chem. Sens. Flavour 2, 51-55


Schrotter, H. (1879) 'Über eine im fuselol enthaltene base' Chem. Ber. 12, 1431-1432

Seeman, F. (1972) 'The membrane actions of anaesthetics and tranquillisers' Pharmacol. Rev. 24, 583-655


Shibuya, T. (1964) 'Dissociation of olfactory neural response and mucosal potential' Science 143, 1338-1340


Shibuya, T. and Shibuya, S. (1963) 'Olfactory epithelium and unitary responses in the tortoise' Science 140, 495-496


Shirley, S., Polak, E. and Dodd, G.H. (1983b) 'Selective inhibition of rat olfactory receptors by concanavalin A' Biochem. Soc. Trans. 11, 760-781


Silver, W.L. and Maruniak, J.A. (1981) 'Trigeminal chemoreception in the nasal and oral cavities' Chemical Senses 6, 295-305


'The use of affinity labels in the identification of receptors' in 'Drug Receptors' Ed. by Pang, H.P. Macmillan Press Ltd. pp 183-191


'Cholinergic nerves stimulate mucociliary transport, ciliary activity, and mucus secretion in the frog palate' Cell Tissue Res. 227, 413-421

Snyder, S.H. and Bennett, J.P., Jr. (1976)

'Neurotransmitter receptors in the brain: biochemical identification' Ann. Rev. Physiol. 38, 153-175


'Odor quality of pyridyl ketones' Chemical Senses 5, 343-357

Squirrell, D.J. (1978)

'A study of olfactory mechanisms' Ph.D. thesis: University of Warwick, Coventry, West Midlands, U.K.


'Comparative morphology of olfactory receptors' in 'Olfaction and Taste III' Ed. by Pfaffmann, C. Rockefeller Press, New York. pp 3-21

Stephens, R.E. (1977)

'Major membrane protein differences in cilia and flagella: evidence for a membrane-associated tubulin' Biochemistry 16, 2047-2058


Stoehr, C. (1893)

J. Prakt. Chem. 47, 439-441 (cited by Calabretta, 1978)

Sturner, J.B. and Howell, S.F. (1936)

'The identification of the hemagglutinin of the jack bean with concanavalin A' J. Bacteriol. 32, 227-237


'Calcium content of frog rod outer segments and discs' Biochim. Biophys. Acta 468, 192-208

Takagi, S.F. (1967)


Takagi, S.F. (1969)


Takagi, S.F. and Shibuya, T. (1960a) 'The "on" and "off"- responses observed in the lower olfactory pathway' Jap. J. Physiol. 10, 99-105


Takagi, S.F. and Yajima, T. (1965) 'Electrical activity and histological changes in the degenerating olfactory epithelium' J. Gen. Physiol. 46, 559-569


Takagi, S.F., Kitamura, H., Imai, K. and Takeuchi, H. (1969a) 'Further studies on the roles of sodium and potassium in the generation of the electro-olfactogram; effects of mon-, di-, and trivalent cations' J. Gen Physiol. 52, 115-130


Tonosaki, K. and Shibuya, F.T. (1979) 'Action of some drugs on gecko olfactory bulb mitral cell responses to odor stimulation' Brain Res. 167, 180-184


Westerman, R.A. and Von Baumgarten, R. (1964) 'Regeneration of olfactory paths in carp' Experientia 20, 519-520


Wosy, L., Metzger, E. and Singer, S.J. (1962) "Affinity labeling - a general method for labeling the active sites of antibody and enzyme molecules" Biochemistry 1, 1031-1039

Wolff, L. (1888) 'Uber acetal- und diacetalamin' Chem Ber. 21, 1481-14814.

Woodruff, M.L. and Bownds, M.D. (1979) 'Amplitude, kinetics, and reversibility of a light-induced decrease in cyclic guanosine 3',5'-monophosphate in frog photoreceptor membranes' J. Gen. Physiol. 74, 629-653


Wright, R.H. (1954) 'Odour and chemical constitution' Nature (London) 172, 831


Wright, R.H. (1966) 'Why is an odour?' Nature (London) 209, 551-554

Wright, R.H. (1968) 'How animals distinguish odours' Science Journal 4, 57-62


Wright, R.H. (1978b) 'The perception of odor intensity: physics or psychophysics?' Chem. Senses Flavour 2, 73-79


Wright, R.H. and Burgess, R.E. (1975) 'Molecular coding of olfactory specificity' Can. J. Zool. 52, 1247-1253


Zisapel, N. and Sokolovsky, M. (1977) 'Affinity labeling of receptors' in 'Affinity Labeling' Ed. by Jakoby, W.B. and Wilchek, M.
Zurer, P. S. (1983) 'The chemistry of vision'
PUBLICATIONS

Preliminary accounts of some of the studies presented in this thesis have been reported as follows:


