RECEPTOR MECHANISMS IN OLFACTION

BY

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Abstract

This thesis is a study of receptor-mediated events occurring in olfaction. Potential enzymes involved in olfactory transduction were investigated using standard biochemical techniques, and an investigation of the fatty acid receptor/s was attempted using psychophysical methods.

1. Other investigators in olfaction have recently demonstrated an odorant modulated adenylate cyclase in the frog. We have shown that tissue preparations from the rat have high levels of the enzyme adenylate cyclase. This activity was stimulated in the presence of odorants by up to two times the basal value. The concentrations of odorants used were in the range expected to be physiologically relevant. Both basal and stimulated adenylate cyclase were inhibited by μM calcium ion. Tissue preparations from brain showed no odorant activation. Guanylate cyclase was present at a tenth the concentration of adenylate cyclase and showed no odorant simulation.

2. There are no studies in the literature characterising the cyclic nucleotide phosphodiesterases in olfactory tissue. The Sutherland criteria require the presence of a cyclic nucleotide phosphodiesterase (PDE) in olfactory tissue, if cyclic AMP is to be a second messenger in olfaction. It is possible that olfactory PDE is directly regulated by a receptor, as is found in vision. It is important, therefore, that the PDE's in olfactory tissue are characterised.

Tissue preparations of the rat and sheep were shown to have high levels of cyclic nucleotide phosphodiesterase. The activities were not stimulated in the presence of physiologically relevant concentrations of odorants; inhibition of these activities by odorants was not significantly different from that of brain homogenates. Characterisation of the phosphodiesterase isoenzymes separated by DEAE chromatography established that they resembled the isoenzymes characterised from other tissues. The evidence presented indicates that olfactory tissue does not resemble visual tissue, which has a receptor-linked phosphodiesterase. All evidence suggests that PDE's in olfactory tissue serve to return cyclic nucleotide concentrations to resting levels after stimulation. The observation that the Type 1 (calcium/calmodulin stimulated) phosphodiesterase is present in high concentrations in olfactory tissue suggests that calcium ion concentration *in vivo* may be an important regulator of phosphodiesterase activity.

3. The threshold values of various short chain fatty acids, of high purity, were determined using human subjects. The results were one order of magnitude greater than found by Amoore (1970) but showed a similar group trend. Descriptive analysis was also performed on these acids by generating a series of descriptors sufficient to discriminate between the acids. Analysis of the results by principal component analysis yielded a three dimensional map that showed the acids clustering into four groups. These four groups could indicate the presence of four receptors. Attempts to demonstrate structure-activity relationships between the thresholds, and descriptive analysis results with various physical and chemical parameters, failed. This was probably due to the interaction of the acids with more than one receptor.

So far, the lack of success in identifying olfactory receptors by researchers has meant that investigations of olfactory receptors have had to be done using psychophysical techniques such as those used in this report. It will probably not be known if these methods are useful for identifying receptors, until the receptors can be purified and characterised biochemically.

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Declaration

Work done in chapter two of this thesis was carried out in collaboration with Steven Shirley, Jane Robinson and Rajindra Aujla.

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Abbreviations

adenylate cyclase	ATP pyrophosphate lyase (cyclising) (E.C.4.6.1.1.1)
ADP	Adenosine 5'-pyrophosphate
AMP	Adenosine 5'-phosphate
ANOVA	Analysis of variance
ATP	Adenosine 5'-triphor phate
ATPase	Adenosine triphosphatase
BSA	Bovine serum albumin
cyclic AMP	cyclic adenosine 3',5'-phosphate
carnosine	β-alanyl-L-histidine
carvone	5-(1-methyl-1-ethenyl)-cyclohex-2-en-1-one
cDNA	complementary deoxyribonucleic acid
cyclic GMP	cyclic guanosine 3',5'-phosphate
cineole	1,3,3-trimethyl-2-oxabicyclo[2,2,2]octane
citronellol	3,7-dimethyloct-6-en-1-ol
CON A	Concanavalin A
cpm	counts per minute (radioactivity)
DEAE	Diethylaminoethyl
DG	1,2-diacylglycerol
DNA	Deoxy-ribonucleic acid
DTT	Dithiothreitol
emM	absorbance of a 1mM solution
EDTA	Ethylenediaminetetra-acetate
EGTA	Ethyleneglycol-bis(aminoethylether)tetra-acetate
EOG	Electro-olfactogram
F	Fisher Distribution
f.i.d.	flame ionisation detector
g	gravity
G protein	Regulatory GTP-binding protein
G_i	Inhibitory GTP-binding protein
G_o	Brain GTP-binding protein
G_s	Stimulatory GTP-binding protein
GABA	Gamma-aminobutyric acid
GDP	Guanosine 5'-pyrophosphate
GDPβs	Guanosine 5'-O-(β-thio)diphosphate
GMP	Guanosine 5'-phosphate
GppNHp	Guanosine 5'(β,γ-imido)triphosphate
GTP	Guanosine 5'-triphosphate
GTPγs	Guanosine 5'-O-(3-thio)triphosphate
guanylate cyclase	GTP pyrophosphate lyase (cyclising) (E.C.4.6.1.2)
IBMX	3-Isobutyl-1-methylxanthine
IMP	Intra-membraneous particle
IP_3	Inositol-1,4,5-triphosphate
IPG	Inositol phosphate-glycan
iso-valeric acid	3-methylbutyric acid

K_d	Dissociation constant
µ (prefix)	micro-
m (prefix)	milli-
M	Molar
min	minute
MDS	Multidimensional scaling
mRNA	messenger ribonucleic acid
n (prefix)	nano-
p (prefix)	pico-
P	Probability
PCA	Principal component analysis
PDE	3',5'-cyclic nucleotide phosphodiesterase (E.C. 3.1.4.17)
PEI	Polyethyleneimine
phospholipase C	phosphatidylinositol-4,5-bisphosphate phosphodiesterase
PI	Phosphatidyl inositol
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PMSF	Phenylmethyl-sulphonyl fluoride
POPOP	1,4-bis-2(5-phenyloxazolyl)-benzene
ppm	parts per million (v/v)
PPO	2,5-diphenyloxazole
QDA	Quantitative descriptive analysis
R^2	Square of the correlation coefficient
S.D.	Standard deviation
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.E.M.	Standard error of estimate of mean value
t½	half life (of radioactive decay)
TLC	Thin-layer chromatography
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
uv	Ultra-violet
V	Volt

1. CHAPTER ONE: GENERAL INTRODUCTION.

The olfactory system is capable of discriminating, with great sensitivity, between a large variety of volatile molecules. Olfaction is very important in evolutionary terms; it is often the most important sense for many animals and is involved with food-gathering and searching for mates.

The importance of the olfactory sense in man has diminished; comparative anatomy reveals the olfactory system to be less well developed than in other vertebrates. However, olfaction is still of importance to us, although it tends to contribute to our lives without us being aware of it. We do not even have an adequate language to describe odours, and so tend to rely on crude analogies. The importance that olfaction still has on our lives is immediately obvious to anyone whose cold has caused a blockage of the airflow past the olfactory system. This causes the sense of smell to be lost temporarily, and results in an immediate loss of enjoyment when eating. Odours can also evoke strong memories from the past, perhaps also being part of the 'déjà vu' experience. The importance of olfaction as a warning sense has persisted in humans. An intense and bad smell, such as the toxic gas hydrogen sulphide, has an odour that ranks with pain in its unpleasantness. The importance of olfaction to our preference for products is highlighted by the presence of perfume in a large variety of household items.

The versatility of the olfactory system creates problems for those trying to understand the biochemistry involved. The stimulus cannot be well-defined in the way that the stimuli for our other senses can, because of the very great number of agonists, and as a consequence, biochemical researchers have tended to concentrate on simpler sensory systems. The last couple of years have, however, seen the emergence of several new research groups working on olfaction. The surge of results that has occurred has been comparable to that seen a few years ago in vision research. Thus, the future bodes well for a biochemical understanding of olfaction.

1.1. ANATOMY OF OLFACTION.

1.1.1. Gross anatomy of the nasal cavity.

Inhaled air passes into the nasal cavity and through the turbinates which provide a convoluted passage serving to warm up the air. The structural part of the turbinates is formed from cartilage, and this is covered by a layer of respiratory or olfactory epithelium. The number of turbinates and the area of olfactory epithelium varies according to species. In amphibians there is only a single sheet of epithelial cells.

Figures 1.1a, b & c show line drawings of the location of the olfactory turbinates in bisected heads from man, sheep and the rat. The nasal septum, which divides the nasal cavity in two, has been removed in all cases. In both man and sheep most of the turbinates is composed of respiratory epithelium, identified by its synchronously beating cilia. Olfactory epithelium occupies a small area indicated by a darker yellow/brown colour close to the cribriform plate and covers parts of the septum also. The colour is due to pigment granules in Bowman's glands and sustentacular cells (Moulton, 1971). In the rat a mixture of both types of tissue is found distributed throughout the turbinate. The anatomy of olfaction is reviewed in detail by Beidler (1971).

1.1.2. Anatomy of olfactory epithelium.

A diagrammatic cross-section of the olfactory mucosa is presented in figure 1.2. The tissue can conveniently be subdivided into two layers, the superficial olfactory epithelium and the deeper lamina propria. Below the lamina propria lies the cartilage of the septum or turbinate.

1.1.3. Columnar epithelium.

This contains three main cell types; the receptor neurones, the sustentacular cells and the basal cells. The epithelium is shown in more detail in figure 1.3.

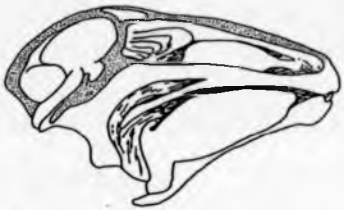
[1] Receptor neurones.

These are bipolar neurones which have a cell body containing the nucleus located deep in the epithelium. Unmyelinated axons pass into the lamina propria. The dendrites of the

Figure 1.1. Sagittal sections of heads from Rat, Sheep and Man. *Ethmoturbinates are shaded in colour.*

1.1a Rat (scale 3:1).

1.1b Sheep (scale 1:3).



1.1c Man (scale 1:4).

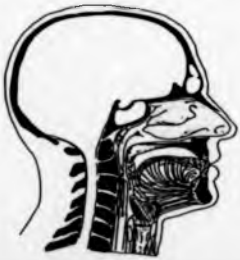
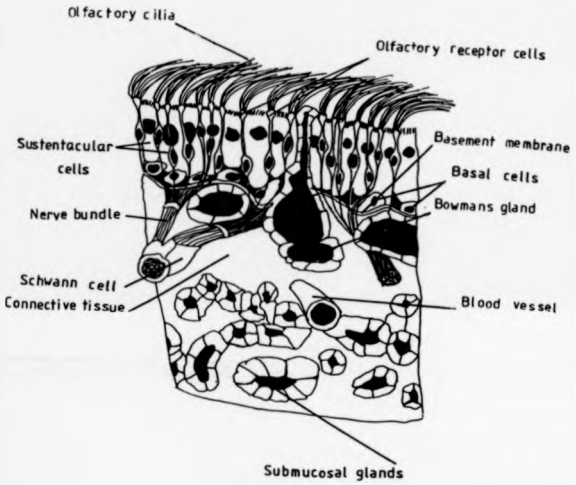


Figure 1.2. Schematic representation of a cross-section of olfactory mucosa showing columnar epithelium and lamina propria (not to scale). Redrawn from Anholt (1987).



neurones continue to the surface of the epithelium where they expand to form an apical knob from which numerous cilia extend. The precise number and length of cilia depends on the species (Menco, 1977, 1980). An increasing body of evidence (see section 1.3.3) indicates that the receptor proteins reside on the surface of the cilia (which increase the surface area), and the apical knob. Olfactory cilia differ from respiratory cilia in that the former lack synchronous beating presumably due to the '9 + 2' microtubule structure being present for only a short distance up the cilia. The cilia lie in the olfactory mucus produced from the submucosal glands, Bowman's glands and possibly sustentacular cells.

[2] Sustentacular cells.

These also extend from the epithelial surface to the basal lamina. The nuclei of the cells lie near the top of the epithelial surface, in contrast to the receptor neurones. The sustentacular cells are presumed to surround and isolate the receptor dendrites. They might also be involved with a structural and nutritive role similar to glial cells in the central nervous system.

[3] Basal cells.

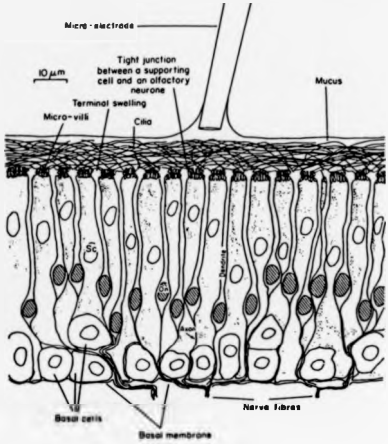
Basal cells lie at the base of the olfactory epithelium. The receptor neurones are exposed to a particularly harsh environment which presumably accounts for the rapid turnover of olfactory receptor neurones. Considerable evidence indicates that the basal cells act as stem cells which mature into receptor neurones and sustentacular cells (reviewed by Farbman, 1986).

1.1.4. Lamina propria.

The axons from receptor neurones pass through the basal cells into the lamina propria. The unmyelinated axons collect as nerve bundles which are sheathed by Schwann cells and then project to the olfactory bulb. Blood vessels permeate the lamina propria, and numerous submucosal glands can be observed. Ducts from Bowman's glands pass up through the olfactory epithelium to the surface.

Figure 1.3. Detail of columnar epithelium.

On, olfactory neurone; Sc, sustentacular cell. Scale: 1mm = 1μm. Taken from Dodd and Squirrel (1980).



1.1.5. Olfactory bulb.

The bundles of axons from the olfactory epithelium pass through perforations in the cribriform plate and into the olfactory bulb. The fine axons are particularly susceptible to damage. Blows to the head can result in the nerves being sheared at the cribriform plate and a consequent loss of smell (anosmia). These axons synapse, with the dendrites of mitral and tufted cells, in structures called glomeruli. The axons from about twenty-six thousand receptor cells converge on each glomerulus which is innervated by about twenty-four mitral and seventy tufted cells (Allison and Warwick, 1949). Mitral and tufted cells are the output cells of the olfactory bulb, their axons pass out of the bulb in the lateral olfactory tract and pass into the olfactory cortex of the brain. Granule and periglomerular cells make synaptic contact with the mitral and tufted cells, and are thought to modulate activity.

1.2. ELECTROPHYSIOLOGICAL STUDIES ON OLFACTION.

The olfactory receptor cells, as discussed previously, are bipolar neurones with dendrites extending up to the surface where they spread out as cilia. The non-myelinated nerve axons extend to the olfactory bulb. As with other fields of biology such as vision, much can be learned from studies of the electrophysiological behaviour of the neurones.

A generalised receptor neurone has a resting potential across its membrane, which is created by the relative concentrations of sodium, potassium and chloride ions inside and outside the cell (called the concentration gradient). The gradient is maintained by various ion-pumps contained in the membrane. Odorants ultimately result in the change of status of the membrane channels which causes a current flow across the membrane. This flow of current changes the membrane potential and results in the so-called receptor potential. The receptor potential spreads passively to an area of membrane with voltage-gated ionophores, (the so-called 'axon hillock'), resulting in the 'firing' of the neurone. A negative resting potential of around -33 to -65mV has been measured by a variety of investigators (reviewed by Getchell, 1986) by the use of intracellular recording techniques in the dendrite. This negative resting potential has been shown to undergo a depolarisation when the neurones are exposed to odorants, consistent with a flow of sodium ions

into the cell.

1.2.1. The Electro-olfactogram (EOG).

The receptor potential of the olfactory epithelium can be observed by the use of electrodes placed just inside the mucus layer. A potential of up to several millivolts can be measured which results from the summed potential of many receptor neurones. The EOG was first seen by Hosoya and Yoshida (1937), and later by Ottoson (1956). The term 'electro-olfactogram' (EOG) was coined by Ottoson due to the similarity of the EOG response to the electro-retinogram in the retina. The typical signal consists of a peak which develops within a second and then drops to an intermediate value because of adaptation. Removal of the stimulus then results in a slow decay to the original signal level. The amplitude of the EOG response is found to be proportional to the logarithm of the stimulus concentration over a certain concentration range. Due to the signal being a summed potential from a large population of cells, the EOG response is not a guide to the responses of individual receptor types. It cannot be readily estimated how many receptor types there are, nor how many respond to a given odorant. Despite the above problems, many studies have been conducted using EOG's with many useful results (reviewed Getchell 1986). Many studies on chemical modification of the olfactory epithelium (using thiol-specific reagents) have been carried out (Getchell & Gesteland, 1972; Menevse *et al*, 1978; Shirley *et al*, 1983). These studies provide evidence that the olfactory receptor is proteinaceous and that several different receptor types are present with broad selectivity. Experiments by Minor & Sakina (1973) and Menevse *et al* (1977) using phosphodiesterase inhibitors have implicated cyclic nucleotides in the transduction process by showing a diminution in the EOG response.

Recently Shirley *et al* (1987 a,b), using the lectin concanavalin A (CON A), have presented evidence that CON A selectively diminishes the EOG response for one class of receptor. The receptor has weak structural specificity and seems to be important for the detection of thiols, carboxylic acids and hydrocarbons of chain length C4 to C6. The selectivity of the CON A effect for one receptor type allowed the authors to estimate the K_d's for several hydrophobic odorants to be of the order of 100nM.

1.2.2. Single unit studies.

Olfactory neurones have extremely small diameter axons of about 0.2μm (Graziadei, 1971) and it is not possible, at present, to make intracellular recordings. It is possible, however, using platinum black metal-filled electrodes, to measure action potentials extracellularly. Many investigators have studied the responses of populations of receptor neurones to a battery of odorants under a variety of conditions, and Getchell (1986) has reviewed much of the research which can be summarised as follows;

[1] Receptor neurones have a relatively low rate of spontaneous activity whose rate of firing can change in response to an odorant. The spontaneous activity can increase, decrease or stay the same when exposed to an odour. Most responses to an odour result in an increase of firing rate.

[2] The response of individual neurones to a battery of odorants indicates that receptor neurones are not specialised to detect one single odorant, but that one neurone usually responds to several different odorants. Surprisingly, no obvious patterns have yet emerged from these studies, indicating a complex system. The results of Sicard & Holley (1984), of the response of a variety of neurones to a battery of odorants, are shown in figure 1.4. The conclusion from studies of this type is that either there are a relatively small number of receptors with a very broad specificity, or each neurone has a large number of different receptors.

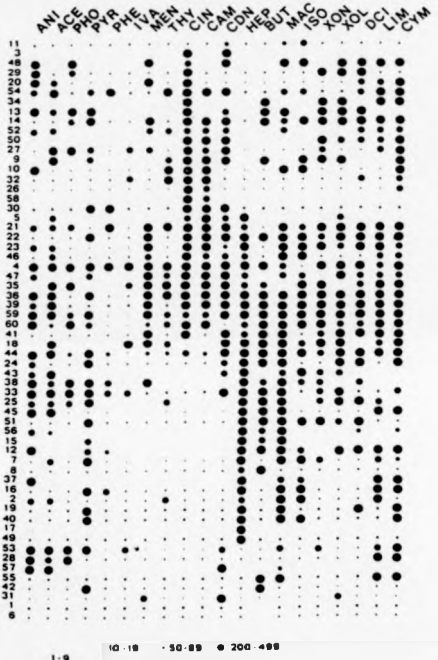
[3] Single units show a concentration-dependent response to increasing odour concentration, with an initial burst of activity followed by adaptation in a similar way as seen for EOG's.

1.2.3. Studies on reconstituted membrane systems and patch clamping.

Odorant-activated conductances formed from mixtures of olfactory homogenates and lipid membrane systems have been demonstrated by Fesenko *et al* (1977), Vodyanoy & Murphy (1983) and Labarca *et al* (1986). The channels all show odorant-activated conductance changes in a reversible manner. Vodyanoy & Vodyanoy (1987) showed a conductance that increased when diethyl sulphide was added at concentrations as low as 10 pM. Cyclic AMP mimicked this

Figure 1.4. Diagrammatic representation of olfactory cell receptor activity following stimulation by odorants (reproduced from Sicard & Holley, 1984).

The spot size is roughly proportional to the spike frequency (spikes/min) measured by extracellular recording from single receptor cells in frog olfactory epithelium after odorant application. Receptor cells are identified by the serial numbers on the left. The odorants tested are anisole (ANI), acetophenone (ACE), thiophenol (PHO), pyridine (PYR), phenol (PHE), isovaleric acid (IVA), l-menthol (MEN), thymol (THY), 1,8-cineole (CIN), ± -camphor (CAM), cyclodecanone (CDN), n-heptanol (HEP), n-butanol (BUT), methylamylketone (MAC), isoamylacetate (ISO), cyclohexanone (XON), cyclohexanol (XOL), (+)-citronnelol (DCI), (+)-limonene (LIM), and p-cymene (CYM).



conductance change when added directly, thus implicating cyclic AMP as a second messenger. No odorant effects were seen in the absence of added ATP and GTP, presumably due to the exhaustion of cyclic AMP in the media. Labarca *et al* (1986) demonstrated an odorant activated cation selective channel activated by nM concentrations of the odorants isobutyl methoxypyrazine and citralva and they argue that their observations support direct activation of their membrane channel by odorants.

More evidence for cyclic AMP as the second messenger involved with channel activation was published by Nakamura & Gold (1987). They managed to patch clamp samples of cilia and demonstrated that a conductance was present. The conductance was gated directly by the action of cyclic AMP and cyclic GMP, and not via protein kinases. This type of channel, up until then, had only been identified in the rod and cone cells of the retina (Fesenko *et al*, 1985). These channels, however, are not identical, as the vision channels are acted upon fairly specifically by cyclic GMP, whereas the olfactory channels are activated equally by both cyclic AMP and cyclic GMP. Stern *et al* (1986) reported that the pharmacological agent l-cis-diltiazem was a specific inhibitor for this channel and could inhibit the electro-retinogram in intact rod cells. The authors suggested that this agent could be a useful diagnostic agent for identifying this conductance measurement. The inhibition of the EOG response by l-cis-diltiazem would provide additional evidence that a directly gated channel is present in olfactory epithelium and is involved with the generation of the EOG response.

More work is required to investigate the inter-relationships between the channels that have been identified in the previous paragraphs. It is possible that some membrane channels may be linked directly to odorant receptors whilst other channels are modulated by second messenger systems.

1.3. STUDIES ON RECEPTORS.

The conventional strategy for biochemical investigations of receptors is to use the binding of a radio-labelled ligand as a 'handle' and then purify, reconstitute and investigate the system.

Researchers in olfaction have met with little success using this method, which could be attributed to the following problems;

[1] An extremely large number of potential agonists complicates studies. Two extreme cases can be envisaged to account for this diversity of agonists; In the first case a relatively small number of receptors will be present with low specificity (but not necessarily low affinity), one would expect a given odorant to bind to several receptors, leading to complications for binding studies. In the second case a receptor could exist for each odorant with analogies to the immune system, which would necessarily result in a vanishingly small concentration of each receptor type.

[2] The hydrophobic nature of most odorants leads to very high levels of non-specific binding which again complicates binding studies, and can totally mask any specific binding.

[3] There is also the difficulty of demonstrating that any binding "seen" has physiological relevance to the detection of odorants. The demonstration of odorant-stimulated adenylate cyclase leads to possibilities of fulfilling this criterion by the use of membrane reconstitution experiments - assuming, of course, that adenylate cyclase couples to the receptor and is regulated by it.

1.3.1. Psychophysical investigation of receptors.

Due to the difficulties discussed above, most investigators have used psychophysics to probe the structure of the receptors. According to Beets (1978):

> "The structure of any molecular species represents, under specific physical conditions, the only source of information carried by the molecule...Consequently, the question whether a relationship between molecular structure and odour exists is meaningless. The only legitimate question is whether it is simple enough to be detected".

Many investigators have attempted structure-activity work on olfaction in an analogous way to other systems; their relative lack of success can be attributed to the following problems:

[1] No *in-vitro* assay exists for olfactory activity, consequently researchers have been forced to

use humans as their assay system. Humans have great difficulty in describing the nature of odours, consequently, writing down a 'consensus' statement of a molecule's odour is nearly impossible.

[2] The lack of an obvious or well-defined 'standard' way of doing such experiments of this kind means that comparisons between different research groups are very difficult.

[3] The process of describing the odour, performed in higher centres of the brain, is far removed in processing terms from the receptors. There is a great deal of convergence from the receptor to the olfactory bulb. The brain may recognise patterns, in which case the reactivity of any particular receptor may not be associated with the overall perceived odour.

[4] An extremely large number of potential odorants exists. Other systems in the body tend to have a single receptor and a small well-defined number of possible ligands. The large numbers of receptors that probably exist may render structure-activity relationships impossible.

Despite these problems many investigators have attempted to deduce some information about the system by studies on humans. J.E. Amoore and many others have attempted to rationalise the detection of odorants. Amoore believed that the three dimensional structure of a molecule was the most important factor and developed his 'stereochemical theory of olfaction' (reviewed by Amoore *et al*, 1964). Amoore and colleagues deduced the structure of a series of receptors from correlations between the shape of molecules and their odour. Subsequent to Amoore's pioneering work many researchers have attempted to make sense of the system. Any theory on the relation between the structure of a molecule and its odour has to take into account the following problems:

[1] Odorants exist that have the same (or similar) odours but different chemical structure.

[2] Odorants exist that have very similar chemical structure but different odour qualities.

[3] Odorants exist that have similar chemical structures and similar odour qualities.

The main criterion that dictates whether or not a molecule is an odorant is its volatility; molecules with more than one functional group tend not to be volatile enough to be an odorant. It

has been generally found that the odour quality of a chemical depends on both its overall shape and its functional group (reviewed by Boelens *et al*, 1983). No simple patterns have yet emerged to permit a good estimate of the odour qualities of a molecule, however, certain classes of molecules tend to have certain odour types, for example esters tend to be fruity (reviewed by Boelens *et al*, 1983). Organic chemists have been shown to be moderately successful in identifying the functional groups of molecules by their smell (Schafer & Brower, 1975).

Amoore (1977) suggested a scheme for identifying the so-called primary odours (drawing an analogy to vision in which three primary colours exist which correspond to three receptors). Amoore argued that identifying people with a defect in smell towards a particular class of odorants would indicate a corresponding defect in a primary receptor and allow the identification of primary odours. It is fairly easy to detect individuals with such a defect (called specific anosmia) by relatively simple threshold tests. He had already shown the presence of a subpopulation having a specific anosmia to the fatty acids with a 'sweaty' smell, the anosmic defect was greatest for iso-valeric acid suggesting that this acid is a primary odour. Amoore and others have subsequently identified several primary odours (reviewed Jennings-White, 1985). The validity of this technique has not yet been shown, and the identification of receptors will probably have to wait until the different receptors can be separated and characterised biochemically.

1.3.2. Odorant binding proteins.

Many odorant binding proteins, from olfactory tissue, have been reported in the literature (K_d's between 10^{-5} and 10^{-10}M) but none, so far, has been shown to be physiologically relevant in the detection of odorants. A camphor binding protein has been demonstrated by Fesenko *et al* (1979) of molecular weight 120,000, an anisole binding protein by Goldberg *et al* (1979) of molecular weight 17,000 to 44,000, and an androstenone binding protein by Gower *et al* (1981) of molecular weight 23,000. Rhein & Cagan (1980) reported on the binding of amino acids to trout membranes. Pelosi *et al* (1982) and Wood & Dodd (1984) demonstrated a pyrazine binding protein. Edwards & Dodd (1987) showed the presence of a nicotine binding protein. The soluble pyrazine binding protein has subsequently been extensively studied by Pevsner *et al* (1985), and

has a molecular weight of approximately 40,000, consisting of two monomers. The protein is found in both respiratory and olfactory tissue and appears localised in mucus and Bowman's glands, suggesting that the protein is secreted into the mucus. Cavaggioni *et al* (1987) reported that sequence analysis of the pyrazine binding protein showed marked homology to a family of proteins, of unknown function, in the urine of male mice and rats, though it is possible that they are involved in the strong behavioural effects produced by rodent urine. Lee *et al* (1987) reported the isolation of mRNA coding for a polypeptide of molecular weight 20,000. The mRNA was localised to Bowman's glands and sequence homology was noted to several serum transport proteins. The presence of the pyrazine binding protein and the protein reported by Lee *et al* (1987) suggests that a family of odorant transport proteins may exist in the mucus, involved with either odorant clearance or concentration.

1.3.3. Tissue preparations.

A proper study of the biochemistry of olfaction requires a suitable tissue preparation. Several workers have attempted to separate out olfactory neurones with the view of undertaking biochemical studies on them either directly or after tissue culture. Only limited success has been demonstrated (reviewed by Hirsch and Margolis, 1981). Schubert *et al* (1985) reported a method for tissue culture that yielded neurone-like cells, presumably derived from basal cells during the culturing. Nakamura and Gold (1987) used a tissue separation technique to separate out olfactory neurones to allow patch clamping of cilia. The difficulties of the technique and the small numbers of recognisable cells has led to alternative techniques being used for biochemical studies of the olfactory mucosa.

Evidence points to the cilia and terminal swellings as the location for receptor proteins and the transduction process (Rhein & Cagan, 1981). The cilia are the first part of the neurones encountered by odorants, their large surface area presents a likely site for the location of the odorant receptors. The enrichment of intramembrane particles in cilia provides evidence implicating cilia as the site for the receptors (Menco *et al*, 1976, see also section 1.3.4). Further evidence is provided by the observations that destruction of the cilia results in loss of the EOG

response; the EOG response can then be observed to return in parallel with the cilia (reviewed Getchell 1986).

The development of a suitable cilia preparation was therefore a priority. The first cilia preparation used in olfaction was reported by Rhein & Cagan (1980), using trout. Their method was based on that of Linck (1973) for the isolation of cilia from the mollusc. The procedure used an EGTA wash followed by a 'shock' of 10mM calcium, causing cilia detachment. Rhein & Cagan (1981) used this preparation to demonstrate amino acid binding in trout, showing the enrichment of this binding in cilia. The procedure was used by Chen & Lancet (1984) and then by Anholt *et al* (1986) on the frog. The observation of the enrichment of potential transductory enzymes and proteins in cilia provided additional evidence for their importance in olfaction. The demonstration of the existence of the odorant-stimulated adenylate cyclase (Pace *et al*, 1985; Sklar *et al*, 1986) implicated cilia as the site of the transduction process (see section 1.4). Attempts to duplicate the preparation in the rat have been less successful, possibly because the rat has much shorter cilia (frog cilia approximately 200μm, rat cilia approximately 30μm, Menco, 1977). An alternative preparation has been published (Shirley *et al*, 1986), in which a light sonication is used to strip off the cilia, albeit in the non-recognisable form of membrane vesicles. The demonstration of the odorant-stimulated adenylate cyclase in comparable yields to the amphibian calcium shock preparation suggests the validity of the alternative tissue preparation. Sklar *et al* (1986) reported the use of the calcium shock in the rat, but gave no estimates of yields which were found by Shirley *et al* (1986) and by Pace & Lancet (1986) to be very low. The cilia preparations discussed above will be suitable for the biochemical characterisation of the olfactory tissue of the frog and rat.

1.3.4. Other biochemical studies of receptors.

Many hypotheses have been proposed as to the nature of the olfactory receptors (for a review see Price, 1981). It is, however, widely believed by most investigators that the olfactory receptors are proteinaceous. The following evidence points towards this view:

[1] The EOG response is inhibited by protein modifying agents and lectins (reviewed by Getchell, 1986).

[2] The presence of specific anosmias, in which a person has a much reduced ability to detect a particular odorant or group of odorants suggests the presence of inheritable groups of protein receptors (Amoore, 1970).

[3] Different enantiomers show differences in perceived odour type, albeit slight. (Freidman & Miller, 1971).

Freeze-fracture studies by Menco *et al* (1976) demonstrated a large number of intramembranous particles (IMP's) located at the base of the cilia, of size 7-11nm. They calculated that 4.3 to 6.0 × 10^3 IMP's μm^{-2} were present on the cilia and about 3.4 × 10^3 IMP's μm^{-2} on the dendritic knob. Each cilium had a surface area of about 160μm^2 with about 10^6 IMP's. Non-sensory cilia had far fewer IMP's (0.3 to 0.8 × 10^3 μm^{-2}). The authors suggested that IMP's represented receptor proteins.

Chen & Lancet (1984) and Chen *et al* (1986) have identified and characterised a protein of molecular weight 95,000 which was enriched in olfactory cilia with respect to other olfactory epithelial fractions (other proteins shown in cilia were not). The protein could not be washed off the membranes, indicating that it was probably an integral membrane protein. Additionally, the presence of glycosylation suggests that the protein might span the membrane. The characteristics of 'gp95', as it is termed by the authors, point to it being an ideal receptor protein. Indeed, calculations by the authors suggest that the density of gp95 correlates with the density of IMP's reported by Menco *et al* (1976). The authors postulate that the relatively broad band on the gel represents a family of receptor proteins, with the presence of constant and variable regions similar to immunoglobulins. Fesenko *et al* (1987) have recently found that a complex of two glycoproteins of molecular weight 88,000 and 55,000, present in rat olfactory epithelium, can bind the odorants camphor and decanal. The authors postulated that the glycoproteins they found were functionally identical to gp95 found by Chen *et al* (1986). Further work is required to determine if these glycoproteins are indeed olfactory receptor proteins.

1.4. TRANSDUCTION PROCESSES

1.4.1. Potential candidates for transduction systems.

The development of suitable olfactory cilia tissue preparations (Shirley *et al*, 1986; Pace & Lancet, 1986; Sklar *et al*, 1986) has allowed investigators to study the possible transduction processes occurring in olfaction.

Previous biochemical studies of transduction processes occurring in other cells have resulted in the following types of transduction systems being identified (these are also summarised in figure 1.5):

[1] Receptor gated ion channel.

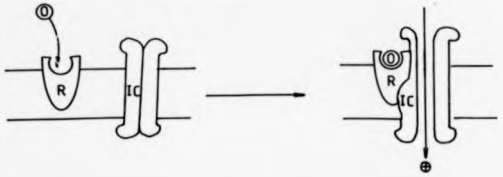
In this system, of which the most famous example is the acetylcholine receptor (reviewed by Changeux & Revah, 1987), the receptor directly controls an ion channel via allosteric changes in the receptor which are produced when the ligand binds. The structure of the ion channel changes, causing the current flow to be altered. Phosphorylation and dephosphorylation of the receptor, via protein kinases, allows for a control of the affinity of the ligand for the receptor and also provides a means of desensitising the receptor and expelling the ligand after the stimulus.

[2] Cyclic nucleotide cascade.

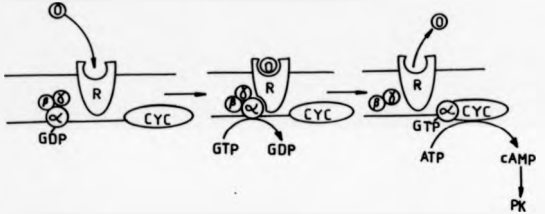
This system is found in many tissues (reviewed Drummond, 1983; Gilman 1984; Schramm & Selinger, 1984) and is usually mediated by either cyclic AMP or cyclic GMP. The receptor controls the activity of the cyclase enzyme via a regulatory GTP-binding protein, either stimulating or inhibiting the breakdown of cyclic AMP (see introduction to chapter 2). Recently a cyclic nucleotide phosphodiesterase has been implicated as a regulator in the rod outer segments in the retina, rather than the cyclase, although a regulatory GTP-binding protein is still present (reviewed by Altman, 1985 and see introduction to chapter 3). The increase in intracellular cyclic nucleotide concentrations then stimulates cyclic nucleotide-dependent kinases (reviewed by Cohen, 1982) which can phosphorylate certain key proteins such as ion channels. Ion channels have been demonstrated that are directly regulated by

Figure 1.5 Possible transduction mechanisms involved in olfaction.

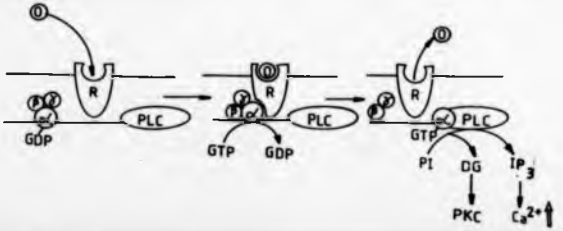
1.5a) Binding of an odorant (O) to a receptor (R) causes a conformational change allowing binding to a ion-channel (IC). Binding of the receptor to the ion-channel opens the ion-channel and allows current flow. Desensitisation could be accomplished by phosphorylation of either the receptor or the ion-channel.



1.5b) Binding of an odorant (O) to a receptor (R) causes a conformational change allowing binding of a GTP-binding protein (α, β & γ). The binding of the GTP-binding protein to the receptor allows exchange of GDP for GTP which causes dissociation of GTP-binding protein. The α subunit then activates an adenylate cyclase enzyme (CYC). Increased intracellular cyclic AMP then activates a cyclic AMP dependent protein kinase (PK).



1.5c) This model is similar to model (b) above except that a phospholipase C is activated (PLC) instead of a cyclase. Breakdown of phosphatidyl inositol bisphosphate (PI) produces diacylglycerol (DG) which activates protein kinase C (PKC) and Inositol trisphosphate (IP_3) which causes a release of calcium.



cyclic AMP and not through a phosphorylation scheme (Fesenko *et al*, 1985). The cyclic nucleotide cascade has the advantage over direct modulation of ion channels in that an additional degree of amplification is present.

[3] Receptor stimulated hydrolysis of inositol phospholipids.

In recent years evidence has been accumulating that the receptor stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C, first noticed by Hokin & Hokin (1953), is an important transduction system in many cells (reviewed by Majerus *et al*, 1986). Approximately 75-85% of the total inositol lipids is composed of phosphatidylinositol (PI), the major phosphoinositide in most cells. Inositol lipids in turn account for 5-10% of the total cellular phospholipids. The receptor mediated hydrolysis of PIP_2 liberates two second messengers inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DG). This receptor mediated breakdown appears to be regulated by a GTP-binding protein (G protein) in a similar way to the adenylate cyclase discussed in the previous section. The G protein appears to be a distinct type from the G protein involved with the cyclase enzyme but has so far remained elusive to study (reviewed by Bourne, 1986). The soluble second messenger inositol-1,4,5-trisphosphate (IP_3) was shown by Streb *et al* (1983), to cause the release of calcium from the endoplasmic reticulum. This increase in intracellular calcium can mediate numerous events via calcium dependent enzymes. The membrane bound second messenger 1,2-diacylglycerol (DG) has been shown to activate the enzyme protein kinase C which can then phosphorylate certain key enzymes in the cell (Nishizuka, 1984). DG is the site of action of phorbol esters which mimic the effect of protein kinase C. The time scale of phosphoinositide breakdown in systems studied so far would seem to be too slow to account for the rapid response to odour (fractions of a second).

Evidence for the preceding three transduction systems will be presented in the following sections. The assumption that only one transduction system can operate in any one particular cell does not seem to be valid any more. It has been shown that some liver cells have both a glucagon

receptor-coupled adenylate cyclase and a glucagon receptor-coupled phospholipase C (reviewed Peterson & Bear, 1986); this allows the possibility of studies being complicated by the two (or more) systems interacting. Possible interactions could result from the rise of intracellular calcium due to inositol trisphosphate release. Calcium can modulate the activities of the cyclases, phosphodiesterases and protein kinases via calcium-binding proteins. Protein kinase C could also phosphorylate the G protein, receptor or cyclase to modulate activity. Conversely, cyclic nucleotide dependent kinases could phosphorylate proteins involved with phosphatidylinositol breakdown and modulate their activity. Possible interactions involved in diabetes are summarised by Pennington (1987).

In addition to the presence of proteins implicated in the above transduction systems, various other proteins and enzymes have been identified in extracts of olfactory mucosa.

A soluble protein of 18-19,000 molecular weight has been found only in mature olfactory neurones and has been termed the 'olfactory marker protein' (reviewed by Margolis 1985). Despite the fact that the gene encoding the protein has been cloned, and the amino acid sequence deduced from the cDNA, the function of this marker protein remains unknown.

Olfactory neurones have been shown to synthesise the dipeptide carnosine (β-alanyl-L-histidine) at high concentrations and it has been suggested that carnosine may be released as a neurotransmitter in the olfactory bulb (Margolis, 1977). Carnosine and the enzymes catalysing its formation were shown by Margolis to be present in higher concentrations in the olfactory epithelium and olfactory bulb than in the central nervous system. There is also evidence that taurine may be a neurotransmitter (Lindquist *et al*, 1983). The presence of muscarinic cholinergic receptors has been demonstrated in olfactory epithelium (Hedlund & Shepherd, 1983). The enzymes for the synthesis of acetylcholine are also present and the authors suggest that acetylcholine may be involved with the control of secretory processes.

Anholt *et al* (1984) have demonstrated the presence of peripheral-type benzodiazepine receptors in the olfactory epithelium, at the highest specific activities so far reported. Anholt *et al* (1986) reported that the peripheral benzodiazepine receptors were associated with the outer

mitochondrial membrane. The function of these receptors is not known at the present time.

Koch (1971) reported that the 13000 ×g pellet from homogenates of rabbit olfactory mucosa exhibited Na-K-ATPase activities which were affected by odorants. Further studies by Dreesen & Koch (1982) reported that these effects were not seen in homogenates of brain tissue and that complex patterns of inhibition and stimulation were seen over a range of odorant concentrations with maximal stimulation seen at 1mM odorant. A variety of odorants showed differing patterns of Na-K-ATPase modulation and different areas of turbinates also gave rise to varying effects. The changes in activity could be due to perturbation of the environment of the enzyme and more work is necessary to determine whether or not the observed effects are significant in olfactory transduction.

1.4.2. Studies on olfactory adenylate cyclase.

Much evidence has now implicated the importance of cyclic nucleotides in the transduction mechanisms of olfaction. Kurihara and Koyama (1972) demonstrated that high levels of adenylate cyclase were present in olfactory mucosa, but reported no effect of odorants on its activity. Minor & Sakina (1973) and Menevse *et al* (1977) showed that membrane permeable cyclic AMP analogues and phosphodiesterase inhibitors resulted in an inhibition of the EOG response in the rat, with cyclic GMP analogues having no effect. Menevse *et al* (1977) investigated the effect of odorants on adenylate cyclase using a tissue preparation but found no effect. The reasons for this are not known, but could have been due to different homogenisation techniques to that used in current preparations, or to the use of sucrose in their preparation which has been shown to stimulate adenylate cyclase activity (Shirley *et al*, 1986).

Many experiments on olfactory adenylate cyclase and its GTP-binding protein (G protein) have been published recently (reviewed by Anholt, 1987 and Lancet & Pace, 1987). Pace *et al* (1985) first reported the major step forward when they demonstrated that odorants could stimulate adenylate cyclase in a frog cilia preparation. They showed that their cilia preparation contained adenylate cyclase with a specific activity fifteen times that of the brain whilst respiratory cilia showed only 1% of the specific activity. Various odorants were tried on their

preparation, and they found variable degrees of stimulation up to 250% of basal values. They showed that odorants acted in an additive way, suggesting that multiple receptors were present and converging on a common cyclase enzyme. No effect of odorants were seen in brain or liver preparations. GTP and its non-hydrolysable analogues guanosine 5'(β,γ-imido)triphosphate (GppNHp) and guanosine 5'-0-(3-thio)triphosphate (GTPγs) along with fluoride ions and forskolin enhanced the basal activity, implicating the presence of a G protein in regulation. An important criterion for the physiological significance of adenylate cyclase is the demonstration of activation in the physiologically relevant range of odorant concentrations. Pace *et al* (1985) demonstrated that an adenylate cyclase dose response was seen over a range of odorant concentrations expected from studies on the EOG response.

Subsequent to this finding, other reports have confirmed and extended these findings. Sklar *et al* (1986), using a similar calcium shock procedure to that of Pace *et al* (1985), confirmed the odorant-stimulated adenylate cyclase in the frog and also reported its presence in the rat. They studied a variety of odorants and obtained varying degrees of stimulation depending on the odorant used. About sixty odorants were investigated and around half of these led to stimulation. They found the fruity, floral, minty and herbaceous odorants tended to stimulate adenylate cyclase, whilst pungent and putrid odorants tended not to significantly increase activity. The failure to observe this stimulation for a significant proportion of odorants suggests that at least one further transduction process is involved in olfaction.

Shirley *et al* (1986) also reported the presence of the odorant-stimulated cyclase in rat preparations. They found the calcium shock cilia preparation to be unsatisfactory for the rat, (confirmed by Pace & Lancet, 1986), and resorted to a light sonication to strip off the cilia. They reported comparable specific activities and degree of stimulation to odours as reported by other researchers. Calcium was found to inhibit both the basal and stimulated adenylate cyclase in the micromolar range. Shirley *et al* (1987c) reported that Arrhenius plots of rat olfactory adenylate cyclase are fairly flat, indicating relative insensitivity to temperature. They also reported that the activity was relatively insensitive to membrane fluidisation. The authors argued that these properties are desirable for a transduction system in such an exposed environment.

The observation that PDE inhibitors and cyclic AMP analogues reduced the EOG response of the rat rather than enhanced it (Minor & Sakina, 1973; Menevse *et al*, 1977) is at odds with the hypothesis that cyclic AMP is a second messenger. Anholt (1987) suggests that an explanation for this could be due to the presence of a receptor directly controlling an ion-channel; a cyclic AMP dependent protein kinase may cause desensitisation of the receptor via phosphorylation (figure 1.6). The stimulation of adenylate cyclase is, therefore, subsequent to the activation of the ion-channel and involved with desensitisation. Cyclic AMP analogues and PDE inhibitors would then be expected to diminish the EOG response. There is not sufficient evidence at the moment to determine which hypothesis is correct.

1.4.3. Studies on olfactory G proteins.

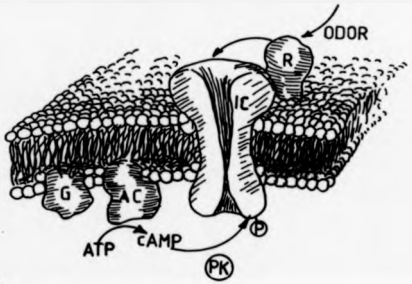
The G protein in the cilia preparations was investigated in more detail by Pace & Lancet (1986) and by Anholt *et al* (1987). Pace & Lancet (1986) reported that cilia preparations showed an enrichment of a polypeptide of 42,000 molecular weight as compared to the rest of the epithelium. This polypeptide underwent [^{32}P]ADP-ribosylation catalysed by cholera toxin, and was tentatively identified as the α subunit of the stimulatory GTP binding protein (G_s). They also report two other polypeptides labelled by pertussis toxin, which they identified as inhibitory G protein (G_i), which is involved with an inhibitory response to adenylate cyclase and brain GTP binding protein (G_o) for which no role has yet been identified. These two polypeptides were also present in respiratory cilia. Anholt *et al* (1987) studied the localisation of G proteins in the olfactory epithelium by the use of subunit-specific antisera. They found three G proteins on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with molecular weights of 45,000, 42,000 and 40,000 corresponding to G_s, G_i and G_o respectively. There was no immunoreactivity against the α subunit of retinal transducin, which tends to rule out the possibility of a receptor-linked PDE as found in vision (Altman, 1985; Stryer, 1986; Stryer 1987). The olfactory cilia were enriched in $G_s\alpha$ relative to $G_i\alpha$ and $G_o\alpha$, compared to the remaining epithelium or respiratory cilia. Immunohistochemistry demonstrated intense staining of the ciliary surface and axon bundles when using antisera against the β subunit. Antisera against the

Figure 1.6. Two possible hypotheses resulting in an apparent odorant modulated adenylate cyclase. Redrawn from Anholt (1987).

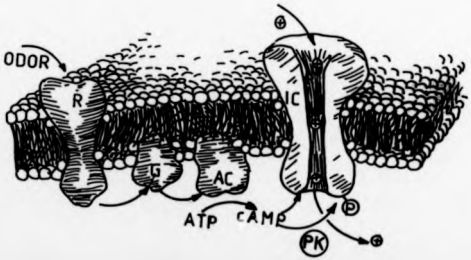
(a) Linkage of receptor (R) to an ion-channel (IC) causes opening of the channel directly. Cyclic AMP dependent phosphorylation by a protein kinase (PK) leads to inactivation of the ion-channel.

(b) Linkage of receptor (R) via a GTP-binding protein (G) to adenylate cyclase (AC) results in the generation of cyclic AMP which, either directly, or via phosphorylation with a protein kinase (PK), activates an ion-channel (IC).

(a). Channel desensitisation via cyclic AMP.



(b). Channel activation via cyclic AMP.



common α sequences stained cell membranes of olfactory receptor cells and Bowman's glands. Additional evidence implicating G proteins in olfactory transduction is the observation that patients with pseudohypoparathyroidism (an inherited deficiency of G_s) have impaired olfactory sensitivity (Weinstock *et al*, 1986).

1.4.4. Effects of cyclic nucleotides on olfactory mucosa.

As explained previously, cyclic AMP usually mediates its effects via a cyclic AMP-dependent protein kinase. Heldman & Lancet (1986) have demonstrated protein kinase activity in olfactory cilia which is activated by both cyclic AMP and GTPγs, though more efficiently by the latter. In respiratory cilia, cyclic AMP-dependent protein kinase was GTPγs insensitive. They also showed the incorporation of labelled [^{32}P] phosphate into several polypeptides. In particular, one band at 24,000 molecular weight was intensely stained and was shown to be located specifically in olfactory tissue.

Nakamura & Gold (1987) report that patch-clamped membranes of cilia have a conductance that is gated directly by cyclic AMP and cyclic GMP with analogies to the system found in the retina (Fesenko *et al*, 1985). This suggests that cyclic AMP may directly gate a channel in the membrane rather than through a protein kinase, allowing the influx of sodium. Additional evidence for the effect of cyclic AMP on membrane channels comes from Vodyanoy & Vodyanoy (1987) who showed that cyclic AMP could mimic the effects of odorants in producing conductivity changes in lipid membranes containing olfactory epithelial homogenates.

1.4.5. Studies on olfactory phosphoinositide breakdown.

The inability of some odorants to stimulate adenylate cyclase suggests that another transduction system may operate in parallel to the odour stimulated cyclase. An obvious candidate is the modulation of phosphatidylinositol turnover (Majerus *et al*, 1986). Huque & Bruch (1986) showed that L-alanine (an odorant for fish) could affect the activity of phosphatidylinositol-4,5-bisphosphate phosphodiesterase (phospholipase C) in fish cilia. The authors interpreted their findings as evidence for this transduction mechanism. The presence of

protein kinase C in olfactory membranes was investigated by Anholt *et al* (1987) and Heldman & Lancet (1986). Anholt *et al* (1987) demonstrated protein kinase C by immunoblot analysis and by phorbol ester binding, however, protein kinase C was also found in cilia from isolated respiratory epithelium. Heldman & Lancet (1986) were unable to demonstrate the presence of protein kinase C activity.

Further work is necessary to investigate this possible transduction system.

1.4.6. Cytochrome P-450.

Recent reports have indicated that the cytochrome P-450 enzyme systems are present in the olfactory mucosa of several different species at concentrations second only to those in the liver (Hadley & Dahl, 1982). The specific activity of the olfactory enzyme was in fact shown by the authors to be greater than that of the liver enzyme. Recently Foster *et al* (1986) have localised the enzyme, by the use of immunocytochemical staining, to the cells of the olfactory epithelium and Bowman's glands; no staining was observed in the nerve bundles in the lamina propria. Gower *et al* (1981) reported the presence of steroid metabolising enzymes in olfactory mucosa which may well have been due to cytochrome P-450 activity.

The reason for the high levels of cytochrome P-450 in olfactory mucosa is subject to much speculation. The drug metabolising enzymes in the liver exist to remove unwanted chemicals in the body by increasing their water solubility. Olfactory mucosa is exposed to an especially harsh environment, with constant bombardment by harmful chemicals, and it is quite likely that the cytochrome P-450 is present to remove these chemicals quickly before they can cause damage. Another potential function for cytochrome P-450 is in the removal of odorants after stimulation. Odorants are usually hydrophobic and would be expected to linger around for a significant time after stimulation. Cytochrome P-450 may help in removal by metabolising odorants to more polar compounds. The metabolism of odorants by cytochrome P-450 might result in the formation of other odorants having different odour characteristics.

Another less likely use for cytochrome P-450 is as part of a transduction system of some sort, the P-450 having been modified from its original function in some way.

The reasons for the location of cytochrome P-450 in olfactory epithelium needs a great deal of further work. However, the surprisingly high concentrations of this enzyme in olfactory epithelium indicates that it must have an important function in olfaction.

1.5. SUMMARY

The ability of the olfactory system to respond to such a wide variety of odorants is very interesting. Evidence from electrophysiological, biochemical and psychophysical experiments have tended to support the hypothesis that a relatively small number of receptors exist which have a broad response to odorants. Processing, by the olfactory bulb and the olfactory cortex of the brain, converts the patterns of reactivity thus obtained to the perceived odour. So far, the olfactory receptors have not been identified, which is mainly due to the lack of any means of labelling such receptors. Many odorant binding proteins have been identified but there is little evidence that any of these are of physiological significance for the detection of odorants.

Suitable tissue preparations have, in the last few years, enabled a re-investigation of the second messenger systems present in olfactory epithelium. The demonstration of an odorant-stimulated adenylate cyclase by several research groups has indicated that olfactory transduction may resemble the 'classic' receptor-coupled adenylate cyclase as found in many other systems. The observation, however, that not all odorants stimulate adenylate cyclase has led to speculation that another transduction system could exist in parallel to the receptor-coupled adenylate cyclase. The search for this other transduction mechanism is continuing but has not been identified as yet. It has been demonstrated that cyclic AMP dependent phosphorylation of certain proteins occurs specifically in olfactory cilia, leading to speculation as to the nature of these targets. Various ion channels have been identified in olfactory epithelium and recently, a cyclic AMP/GMP stimulated channel has been demonstrated in olfactory cilia which had previously only been identified as a component of the visual transduction mechanism.

The method of removal of the odorants from the olfactory epithelium after stimulation has always been a mystery. A possible candidate for this role is cytochrome P-450 which has been

found in surprisingly high concentrations in olfactory epithelium.

The large number of recent biochemical studies of olfaction has produced much evidence for the sequence of events that occur between the binding of an odorant to a receptor and the depolarisation of the receptor neurone. The next few years will, hopefully, see many of the questions answered that now exist about olfactory transduction. Understanding of the olfactory transduction mechanism may then pave the way for identification and investigation of the elusive olfactory receptors.

2. CHAPTER TWO: THE ODORANT-STIMULATED ADENYLATE CYCLASE OF THE RAT.

2.1. INTRODUCTION.

Cyclic nucleotides are important second messengers. Hormone or neurotransmitter-sensitive adenylate and guanylate cyclase activity has been demonstrated in many cell types (Drummond, 1983; Gilman, 1984; Schramm & Selinger, 1984). The change of cyclic nucleotide concentration in the cell that results from modulation of the cyclase by the ligand can then regulate the activity of cyclic nucleotide-dependent protein kinases. The protein kinases can then in turn regulate the activity of a whole variety of cellular proteins (Cohen, 1982). Alternatively the cyclic nucleotides can directly regulate the activity of certain ion channels found in rod cells and olfactory epithelium (Applebury, 1987).

2.1.1. The components of the adenylate cyclase system.

The complete adenylate cyclase system, involved with the trans-membrane signalling function, can be considered as a high molecular weight complex consisting of a receptor facing outwards from the cell membrane, a GTP-binding regulatory protein (G protein) and the adenylate cyclase catalytic unit, embedded in the inner membrane surface. The properties of the three components in the system are summarised in the following three sections:

2.1.1.1. Receptor.

The receptor provides the specificity of the system and a variety of ligands have been shown to affect the activity of adenylate cyclase (Drummond, 1983; Gilman, 1984; Schramm & Selinger 1984). Ligands causing stimulation of adenylate cyclase include the β-adrenergic agents, adrenocorticotropin, gonadotropins and many others. Ligands causing inhibition of adenylate cyclase include α-adrenergic agents, muscarinic agents, opioids and others. Agonists bind with higher affinity to the complex of receptor/G protein than to the free receptor and addition of

guanine nucleotides reduces the affinity of agonists for the receptor, presumably by inducing dissociation of the G protein from the receptor (Lefkowitz *et al*, 1983). Complex binding kinetics can be an indication of a receptor coupling to a G protein.

Desensitisation of the system can arise from receptor sequestration from the cell surface, resulting in a reduction of receptor concentration. Phosphorylation of the receptor by protein kinases can label the receptor for this sequestration or can directly alter the affinity of the receptor for its agonist or G protein (Sibley & Lefkowitz, 1985).

The first receptor purified to homogeneity was the β-adrenergic receptor which had a molecular weight of 60,000 (Lefkowitz *et al*, 1983). It was demonstrated that purified receptor could be added back to cell membranes containing the adenylate cyclase and G protein, but lacking a receptor, and that a fully functional system would then result (Cerione *et al*, 1983). The amino acid sequence has been recently determined for the β-adrenergic receptor (Yarden *et al*, 1986; Dixon *et al*, 1986) and for the muscarinic acetylcholine receptor (Kubo *et al*, 1986). The amino acid sequence of rhodopsin has also been determined (Ovchinnikov, 1982). Rhodopsin interacts with a G protein but the effector enzyme differs in being a cyclic nucleotide phosphodiesterase rather than a cyclase (Stryer, 1986). The three sequenced proteins show remarkable homologies in their sequence and structure (Hall, 1987). Each protein seems to have seven hydrophobic regions of 20-25 amino acids which presumably span the membrane, a long C-terminal hydrophilic sequence, a shorter N-terminal hydrophilic sequence and a long loop between the probable transmembrane segments V and VI. These similarities are not surprising when it is considered that all three proteins interact with similar G proteins. It is possible that the present diversity of receptors interacting with G proteins results from mutation of an original evolutionary precursor. Evidence supporting this hypothesis has come just recently with the discovery that a family of ligand gated ion-channels also exists; there are observed similarities between glycine, GABA, nicotinic acetylcholine and calcium channels (Stevens, 1987).

2.1.1.2. G proteins.

G proteins are rapidly being identified as a family of proteins mediating a whole host of responses by cells to extracellular signals. The function of G proteins in coupling receptors to adenylate cyclase is well known (Drummond, 1983; Gilman, 1984; Schramm & Selinger, 1984). A G protein called transducin has been shown to couple rhodopsin to a cyclic GMP phosphodiesterase in vision (Stryer, 1986). It seems likely that G proteins may couple receptors directly to ion-channels (Dunlap *et al*, 1987) and are involved in the receptor stimulated hydrolysis of phosphoinositides (Michel, 1986). The isolation of cDNA sequences coding for the α subunits of the G proteins, discussed below, has allowed a comparison of sequences which has shown extensive homology (40-60%) between the various genes (Spiegel, 1987). Screening tissues with cDNA probes has also identified various other, as yet, unidentified G proteins. The presence of families of G proteins has been recently reviewed (Bourne, 1986; Spiegel, 1987) and the following types of G protein have been identified so far:

[1] G_s.

G_s is involved in the activation of adenylate cyclase by ligands and is found in almost all cell-types of vertebrates. G_s can easily be removed from the membrane with appropriate detergent containing buffers and purified to homogeneity (Northup *et al*, 1980). The protein was found to consist of three subunits termed α, β and γ with molecular weights of 45,000, 35,000 and 10,000 respectively shown by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions. Under non-denaturing conditions the β and γ subunits remain associated but the α subunit readily dissociates when the G protein is activated *in vitro*. The G protein has sub-micromolar affinity for guanine nucleotides and in its α/β/γ form has a bound GDP molecule. It is presumed that binding of an agonist to the receptor allows the G protein to bind with greater affinity. Binding of the G protein to the receptor then results in exchange of the bound GDP for GTP. The G protein then probably dissociates into α and β/γ subunits which then separate from the receptor. The separated α subunit can then activate adenylate cyclase. The intrinsic GTPase activity of the

α subunit cleaves the GTP to GDP which allows reassociation of the G protein complex and deactivation of the adenylate cyclase (see also figure 1.5 in chapter 1). It is widely assumed that the β/γ complex is an anchor in the membrane for the hydrophilic α subunit but can also be viewed as an inhibitor of the α subunit and as a necessary component to allow binding of the G protein to the receptor (Gilman, 1984).

The α subunit of the G_s protein is the site of action of cholera toxin which causes ADP-ribosylation of the the α subunit resulting in an inhibition of the GTPase activity and a consequent increase of cyclase activation. Also causing increases in cyclase activation, by interactions with the G protein, are the non-hydrolysable analogues of GTP such as guanosine 5'-O-(γ-thio)triphosphate (GTPγS) and 5'-(β, γ imido)triphosphate (GppNHp) as well as the fluoride ion. GDP analogues such as guanosine 5'-O-(β-thio)diphosphate (GDPβS) cause inhibition of adenylate cyclase activity.

[2] G_i

G_i also modulates the activity of adenylate cyclase but results in an inhibition of activity rather than stimulation. This protein has also been purified to homogeneity (Bokoch *et al*, 1983) and again consists of subunits α, β and γ of molecular weights 41,000, 35,000 and 10,000 respectively. The α subunit differs from that of G_s but the β and γ subunits seem to be identical. G_i is also present in nearly all cell membranes but at a concentration of approximately ten times that of G_s (Gilman, 1984). The mechanism of adenylate cyclase inhibition by G_i has not yet been clarified, but is not just a simple inhibition of the cyclase by the α subunit. Purified β/γ subunits are more effective as inhibitors of adenylate cyclase than the α subunits and Gilman (1984) postulated that the inhibition of adenylate cyclase is due to a direct effect by the released β/γ complex on G_s, resulting in an inhibition of dissociation. Further work is required to determine the mode of action of G_i. G_i also differs from G_s in that it is a substrate for ADP-ribosylation by pertussis toxin rather than cholera toxin which results in an uncoupling of the G protein from the receptor.

[3] Transducin.

Transducin is the G protein, so far found only in the retina, involved with the transduction process in both rod and cone cells (Stryer, 1986). Transducin was purified to homogeneity by Baehr *et al* (1982) and was shown to consist of α, β and γ subunits of molecular weight 39,000, 36,000 and 8 000 respectively. The β/γ complex of transducin differs slightly from that of G_s and G_i in that detergents are not necessary for solubilisation. Transducin couples the receptor, rhodopsin, to a cyclic nucleotide phosphodiesterase in a similar manner to that of G_s to adenylate cyclase. Transducin is a substrate for ADP-ribosylation by both cholera toxin and pertussis toxin.

[4] G_o.

G_o is another G protein that is localised to specific regions. It is found mainly in the brain, is much more abundant than G_i, and accounts for about 1% of the total cerebral cortical plasma membrane protein (Gierschik *et al*, 1986). The β/γ complex appears identical to that of G_s and G_i but has a different α subunit. G_o is a substrate for ADP-ribosylation by pertussis toxin. No function for G_o has yet been identified but its similarity to other G proteins suggests that it has a role in signal transduction and is possibly involved in the regulation of phosphoinositide metabolism (Michel, 1986; Worley *et al*, 1987). There is also some evidence that G_o is involved in the regulation of calcium channels in neurones (Hescheler *et al*, 1987).

In addition to the family of G proteins discussed above there are also the EF-Tu bacterial proteins involved with protein synthesis which have sequence homology to the α subunits of G proteins (Bourne, 1986). These EF-Tu proteins have a cycle of GTP to GDP exchange resembling that of the G proteins.

The mammalian *ras* oncogenes encode closely related proteins of about 21,000 molecular weight. These proteins, which are involved in the regulation of cell division, show similarities to G proteins in that they bind guanine nucleotides, show GTPase activity and are associated with the cytoplasmic surface of the cell membrane (Gibbs *et al* 1985). Though no interaction with β/γ

subunits has been shown the *ras* protein products may be involved with the signal transduction of growth factors.

2.1.1.3. The catalytic unit.

Adenylate cyclase is the only known enzyme activity that can convert ATP to the 3',5' cyclic monophosphate. The cyclase requires magnesium for optimal activity as the substrate is MgATP. The cyclase is essentially inactive in the absence of G_s which enables the cyclase to perform as a useful assay for G_s. The *cyc*⁻S49 lymphoma cell line shows no stimulated cyclase due to the lack of G_s activity but retains the cyclase catalytic unit. The *cyc*⁻ mutant was used in the discovery of the G protein (Bourne *et al*, 1982).

Due to the problems of lability and hydrophobicity very little is known about the catalytic unit relative to the receptor and G protein. It is only recently that the cyclase has been purified to homogeneity by use of a forskolin affinity column (Pfeuffer *et al*, 1985). The cyclase consists of a glycoprotein of a molecular weight of about 130,000. Forskolin is a diterpene which can activate adenylate cyclase in the absence of G_s and seems to have a binding site on the catalytic unit (Seamon *et al*, 1984). The adenylate cyclase can also be regulated by calcium via the calcium binding protein calmodulin (Bender & Neer, 1983).

2.1.2. Relevance of cyclic nucleotides to olfaction.

The recent discoveries of an odorant-stimulated adenylate cyclase and the presence of G_s, G_i and G_o in olfactory epithelium, as reviewed in the introduction, has provided important evidence that cyclic nucleotides are second messengers in olfactory transduction. The discovery of an ion-channel in cilia that is regulated directly by cyclic AMP and cyclic GMP (Nakamura & Gold, 1987) would seem to complete a potential transduction process (see figure 1.6b in chapter 1). The presence of the odorant-stimulated adenylate cyclase at last provides a handle that can be used to purify the presumed receptor proteins. Up until now, any odorant-binding protein that has been isolated cannot be shown to be relevant for the detection of odorants. The ability to assay receptor proteins by adding them back to membrane vesicles containing the G protein and the

cyclase allows a 'handle' during purification (Klausner *et al*, 1984). The discovery of a family of receptors that interact with G proteins (Hall, 1987) gives clues to the possible nature of the olfactory receptors. The discovery that only about half of a series of odorants stimulates adenylate cyclase (Sklar *et al*, 1986) must, however, introduce a note of caution into the use of adenylate cyclase as an assay for receptor reactivity until the reasons for the partial response are known.

2.1.3. Aims.

During late 1984, when it was decided to re-investigate the role of adenylate cyclase in olfaction, a personal communication from Doron Lancet of the Weizmann institute in Israel alerted us that he had succeeded in demonstrating an odorant-stimulated adenylate cyclase in the frog. The Weitzmann group had used a cilia preparation that they had earlier reported in the identification of possible receptor proteins (Chen & Lancet, 1984). Similar studies by our group were hampered by the fact that this cilia preparation did not work very well on the rat due to the much shorter cilia. Only very poor yields had been obtained (Robinson, C.J., personal communication). Due to the importance of the rat as an experimental animal and the need to demonstrate the presence of an odorant-stimulated adenylate cyclase in mammals we decided to attempt to study the adenylate cyclase in rat using a crude preparation of cilia obtained by sonication.

2.2. EXPERIMENTAL.

2.2.1. Materials.

[8-^{3}H] cyclic AMP (23.6Ci/mmole), [8-^{3}H] cyclic GMP (15Ci/mmole), [α-^{32}P] ATP (410Ci/mmole) and [α-^{32}P] GTP (410Ci/mmole) were obtained from Amersham International (Bucks., U.K.). Dowex 50WX8-400 and chromatographic grade alumina, neutral, type WN-3 were obtained from Sigma. All other biochemicals were obtained from Sigma Chemical Company (Poole, Dorset, U.K.) and were of the highest purity commercially available except

imidazole. Odorants were obtained from the Aldrich Chemical Company Ltd (Gillingham, Dorset, U.K.), were of the highest purity available and were not further purified. Columns for the cyclase assay were 'Econo-columns' obtained from Bio-Rad (Watford, Herts.).

2.2.2. Tissue preparation.

Male Wistar rats, approximately 300g in weight were used. The rats were allowed food and water *ad libitum* prior to the experiment. Due to the possibility of non-volatile chemicals stimulating odorant receptors when applied directly in solution it was decided to avoid the use of organic buffers and sucrose, in addition, proteolysis inhibitors and thiol reagents were avoided due to their obvious odour.

The rats were stunned and killed by cervical dislocation and the olfactory turbinates removed after sagittal section of the head. A set of turbinates from a rat was usually 50-100mg in weight. The turbinates were washed three times in buffer A (0.9% NaCl, 1mM EDTA, 5mM phosphate buffer pH 7.0) to remove blood and other debris. The turbinates were then suspended in 10 volumes of buffer B (0.9% NaCl, 5mM phosphate buffer pH 7.0) on ice and sonicated in an MSE 100 W sonicator fitted with an exponentially tapered probe of 3mm tip diameter, for 5 seconds at an indicated power level of 12 microns peak to peak. The suspension was then removed and the turbinates washed with a further 10 volumes of buffer B. The extracts were pooled and centrifuged at 1,000×g for 30 minutes at 4 °C. For some experiments the supernatant was removed and used directly. For the other experiments the supernatant was centrifuged at 20,000×g for 40 minutes at 4 °C. The pellet was resuspended in about 2 volumes of buffer B based on the original turbinate mass.

Protein concentrations were estimated by using the Hartree variation of the Lowry method (Hartree, 1972) using bovine serum albumin fraction V (96-99%) from Sigma as the standard. Approximately 0.4 to 0.6 mg of protein was obtained per rat in the membrane pellet.

Whole rat brain was removed, chopped up, and then prepared in the same way as the olfactory tissue.

2.2.3. Cyclase assay.

The assay for adenylate and guanylate cyclase activity was based on the two step assay procedure developed by White & Karr (1978). The method uses ^{32}P labelled ATP and GTP. Substrate degradation during the assay is minimised by the use of a nucleoside triphosphate generating system, namely the creatine phosphate/creatine kinase reaction. Product degradation is minimised by the use of a phosphodiesterase inhibitor, isobutyl methyl xanthine (IBMX) and the addition of unlabelled cyclic AMP and cyclic GMP. The cyclic nucleotide products are separated from the nucleotide triphosphate starting material by the use of sequential chromatography using an cation exchange resin (Dowex 50WX8-400) followed by alumina. The ion-exchanger is used to remove radiochemical impurities whilst the alumina column retains all nucleotides other than cyclic AMP and cyclic GMP at neutral pH. This separation technique is an improvement over earlier assays using just alumina; these had higher blanks due to the alumina not retaining some radiochemical impurities.

Two perspex racks were made, capable of holding 72 tubes with the ability to place one rack on top of the other to speed the separation procedure. All columns were set up using 'Econo-columns' which were 10mm by 40mm with a 9ml reservoir. Alumina columns were set up using 1g of alumina and were washed with 10mls of water; the alumina was used only once. 1ml of settled Dowex 50WX8-400 was used in each column. The H^+ form was used and the resin was washed successively with 10 ml of each of the following: H_2O, 5% NaOH, H_2O, 6.3% HCl, H_2O.

The reaction mixture for the assay comprised the following components and totalled 80µl in volume;

(1). 25µl of IBMX was made up in water to give an assay concentration of 1mM. In addition odorants were added at this point.

(2). 15µl of assay buffer was added at this point. Tris.Cl, Tris.maleate, arsenate or phosphate buffers were used to give 50mM and pH 7.6 in the assay.

(3). 15µl of the various nucleotides were added at this point. The following components

were dissolved in assay buffer to give the appropriate concentrations in the assay: ^{32}P GTP or ^{32}P ATP (300nCi), ATP or GTP (1 mM), GTP for adenylate cyclase (10μM), cyclic AMP or cyclic GMP (3mM), creatine phosphate (15mM), creatine kinase (100 units/ml, 1 unit=1μmole/min), 0.1mg of bovine serum albumin and $MgCl_2$ (5mM).

(4). 25μl of tissue extract was added to start the reaction and the tubes were vortexed. The sample was used undiluted for most studies.

The tubes were incubated at 30°C for exactly 40 minutes (or other appropriate times) and then the reaction was stopped by adding 150 μl of 1M perchloric acid ($HClO_4$) followed by vortexing. In order to estimate recoveries at the column separation stage, 300μl of water containing about 10,000 cpm of tritiated cyclic AMP or GMP was added at this point. The reaction tubes were then spun at 500xg for 30 minutes in a clinical centrifuge, to pellet the denatured protein and the supernatants applied to the Dowex columns and allowed to drain in. The separation steps for the two cyclic nucleotides differed slightly. For the separation of cyclic AMP two lots of 2.5ml of 10mM $HClO_4$ were added and allowed to drain through, to remove impurities which would raise blank values. The product was eluted into the alumina columns with a further 7 ml of 10mM $HClO_4$. The alumina columns were drained and washed with 10 ml of water followed by 1 ml of imidazole buffer (0.2M, pH 7.1), and the product eluted into scintillation vials using a further 3 ml of imidazole buffer. For the separation of cyclic GMP the Dowex columns were washed with 2.5 ml of 50 mM HCl to remove contaminants and then the products were eluted onto the alumina columns using a further 3.5 ml of 50 mM HCl. The Dowex columns were treated in the same manner as for the cyclic AMP separation.

18 ml of scintillation cocktail (8.3 g PPO, 0.33 g POPOP, 667 ml of Triton X-100 and 1L xylene) were added to the 3ml of product obtained from the above steps. The specially prepared cocktail was necessary to solubilise the high proportion of water used in the scintillation vial. The vials were then counted in a Packard Tri-Carb with windows set to optimal values for the samples. All results were corrected for overspill from the ^{32}P channel to the tritium channel, for quench and for decay of the ^{32}P isotope during the counting process ($t_{1/2}$=14.3 days). The counts

in the tritium channel allowed for correction of recovery during the column separations which was usually of the order of 50%. Blanks from the separation were normally about 10 cpm with experimental values of several hundred cpm (usually about 0.1% of added counts). Activities were usually 5 to 20 pmoles/min per assay. Blanks containing boiled enzyme preparations were equivalent to those containing no enzyme. Results were converted into units of pmoles/min/mg protein or pmoles/min/g tissue using the specific activity of the labelled isotopes after correcting for the decay of the isotope.

2.3. RESULTS.

The olfactory tissue preparation used in the methods section yielded adenylate cyclase activity of typically 500 pmoles/min/mg protein for the resuspended membrane fraction. Preliminary results in Table 2.1 show that the activity was stimulated in the presence of the odorant 1-8-cineole by about 50%, both the stimulated and basal activity showed an increase in activity of about 20% indicating the likelihood of the involvement of a GTP-binding regulatory protein (G protein).

Cineole concentration	GTP concentration	
	Zero	10μM
Zero	371 ± 1	470 ± 34
1mM	530 ± 56	620 ± 8

Table 2.1. Effect of added GTP and the odorant 1-8-cineole on olfactory adenylate cyclase activity.
Crude sonicate was used for the experiment with pH 7.6 phosphate buffer. Activities are in units of pmoles/min/g tissue and are the mean of duplicate experiments with standard deviation.

An attempt was made to separate the high level of basal activity from the stimulated activity, the rationale being that much of the basal activity could come from the epithelium

underlying the cilia and may have different vesicle sizes after sonication. It was found that basal activity sedimented with the stimulated activity and that activity was only associated with the particulate fraction. Most of the activity sedimented between 3,000 and 10,000xg. The varying sizes of vesicle produced by sonication would probably account for the sedimentation of activity over a wide range of g values.

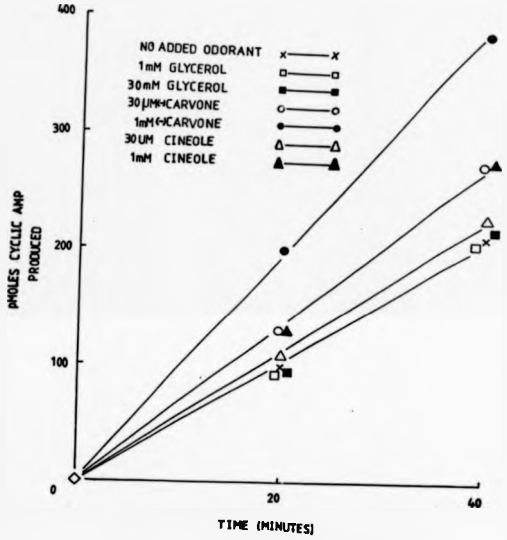
Figure 2.1 shows a comparison of the effects of the odorants 1-8-cineole and (-)-carvone at two concentrations along with a control of glycerol which has no perceived odour (but is volatile). The results indicate that the stimulation of adenylate cyclase by odorants depends on the odorant and its concentration and that the production of cyclic AMP is linear with time up to 40 minutes under the experimental conditions. The non-odorous compound glycerol did not stimulate adenylate cyclase when present at a concentration of 30mM. Non-volatile compounds do not usually smell as they cannot gain access to the olfactory epithelium. It is quite likely that many additional compounds will activate odorant receptors when they have access to receptors in the *in vitro* experiments. The choice of 'control' odorants is therefore very difficult, as it is necessary to use volatile compounds that have no odour whilst bearing in mind that a compound that has no odour to us may smell to the experimental animal. Glycerol was chosen as a suitable control and, as shown in figure 2.1, showed no observable stimulation. The two odorants show quite different degrees of stimulation with 1mM carvone resulting in a 100% increase in basal activity, whereas cineole produced an increase of adenylate cyclase activity only about half that of carvone.

Guanylate cyclase activity was also present in the olfactory preparations but was present at only about 2 to 5 pmoles/min/mg protein. Homogenates from rat brain gave an adenylate cyclase activity of 20 to 30 pmoles/min/mg protein with about 10 times that pelleted by the low speed centrifugation. Both olfactory guanylate cyclase and adenylate cyclase of the brain showed no stimulation of activity when 1mM acetophenone, (+)-carvone or (-)-carvone were added.

It was anticipated that the buffer used in the assay would influence the properties of the cyclase. Organic buffers were avoided as these might be expected to stimulate the cyclase. Three

Figure 2.1. Effects of odorants and glycerol on olfactory adenylate cyclase.

The supernatant from the low speed centrifugation of an olfactory sonicate was incubated with or without the compounds shown on the figure for varying times. The points are the mean of duplicate determinations on a single preparation with a standard deviation of 4%. All compounds gave zero cyclic AMP production at zero time. Units are in pmoles cyclic AMP produced. Redrawn from an unpublished diagram by S.G. Shirley.



inorganic buffer systems were used and the results are presented in table 2.2.

	Buffer		
	Tris.Cl	Phosphate	Arsenate
Basal component	1090 ± 139	1013 ± 106	827 ± 21
Stimulated component	643 ± 132	537 ± 126	673 ± 34
Stimulated/Basal	0.59	0.53	0.81

Table 2.2. Experiment to show the effect of buffers on the odorant-stimulated adenylate cyclase.
All buffers are at 50mM and pH 7.6 in the assay. Crude olfactory sonicate was used for the experiment. Activities are in units of pmoles/min/g tissue and are the mean of duplicate determinations with standard deviation.

The results shown in table 2.2 indicate that the three buffers used have slightly varying effects on the odorant stimulation of olfactory adenylate cyclase activity. Arsenate buffer shows the greatest ratio of stimulated to basal activity, but this buffer caused loss of activity of the cyclase after storage and therefore was not used. The odorant-stimulated adenylate cyclase activity was stable when frozen and stored at -20°C in phosphate buffer. A slight increase in cyclase activity was usually measured on thawing.

Figure 2.2 shows the effect on basal adenylate cyclase activity of several odorants and other compounds. The odorants that have been used in the first section show variable responses with acetophenone giving the highest stimulation. The odorants citronellol and decanoic acid fail to stimulate the cyclase. The sonication procedure would be expected to produce membrane vesicles. As the inside of the vesicle will be isolated from the reagents present outside one would expect that the adenylate cyclase activity that is visible will result from inverted vesicles. The lipophilic odorants would be able to gain access to the receptors inside the vesicles but one might expect that ionised odorants such as decanoic acid and triethylamine would be unable to reach

them. The lack of stimulation by decanoic acid and the slight stimulation seen by triethylamine may be a consequence of this lack of access. Sucrose showed a 200% stimulation of the basal activity; above this level the effect of odorants were obscured. The stimulation by sucrose could be due to impurities in the sucrose or to the compound itself.

The second part of figure 2.2 shows the dramatic effect of GTP and GDP analogues on the adenylate cyclase activity. The non-hydrolysable GTP analogues produce activation of approximately 300% over the basal activity which provides evidence for the involvement of a G protein. In addition the GDP analogue produced the expected inhibition of activity. The slight stimulation by GTP, and the stimulation by fluoride is again consistent with the hypothesis of a G protein. The failure of the two adenylate cyclase agonists histamine and isoproteranol to stimulate adenylate cyclase activity argues against a hormonally controlled adenylate cyclase being present.

Figure 2.3 shows the effect of additional calcium ion concentration on both basal and stimulated adenylate cyclase. Both components were inhibited and the addition of EGTA resulted in an increase of activity of both components. The concentration of calcium in the tissue is not known. Further experiments by Shirley & Robinson (Shirley *et al*, 1986) using calcium buffers showed inhibition of activity from 10^{-7}M to 10^{-3}M calcium.

2.4. DISCUSSION.

The experimental results provide good evidence of the presence of an odorant-stimulated adenylate cyclase in rat olfactory tissue with similar properties to the frog adenylate cyclase reported by Pace & Lancet (1985). The lack of effects of odours reported by Menevse *et al* (1977) is not surprising as the authors used sucrose in their membrane preparation technique (Dodd, G.H., personal communication). The effect of sucrose shown in this report probably resulted in a masking of any further odorant-mediated effects. Kurihara & Koyama (1972) reported no attempts to stimulate their adenylate cyclase activity with odorants.

The rat odorant-stimulated adenylate cyclase has subsequently been confirmed by Sklar *et al* (1986) who used the calcium shock method of cilia detachment but gave no estimates of yield.

Figure 2.2. Olfactory adenylate cyclase activity under a variety of conditions.

The resuspended pellet from an olfactory sonicate was incubated in the presence of the compounds shown. The 100% reaction rate is defined as the activity with no added odorants in the presence of 10μM GTP. Sections 'a', 'b', 'c' represent three different tissue preparations with the following 100% reaction rates: 520pmoles/min/mg protein, 800pmoles/min/mg protein and 440 pmoles/min/mg protein respectively. Tissue preparations 'a' and 'c' are in phosphate buffer and tissue preparation 'b' is in tris-maleate buffer. The results are the mean of duplicate determinations with a standard deviation of 4%. Redrawn from an unpublished diagram by S.G. Shirley.

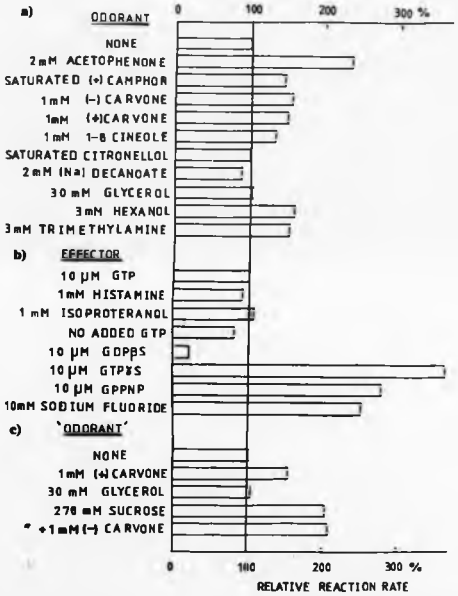
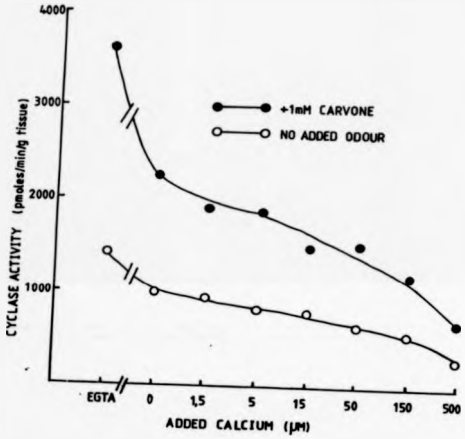


Figure 2.3. Effect of added calcium on olfactory adenylate cyclase activity.

The supernatant from the low speed centrifugation of an olfactory sonicate was incubated in the presence of additional calcium or 100μM EGTA. The effects were investigated on both basal activity (no added odorants) and stimulated activity (1mM (-)-carvone). The results are the mean of duplicate determinations with a standard deviation of 4%.



Robinson, C.J (unpublished results) found that this method gave very poor yields, an observation that was confirmed by Pace & Lancet (1986). The method of cilia preparation used in this study whilst not yielding recognisable cilia seems to result in a cyclase activity comparable to the other method and the assumption that it strips of mainly the cilia would seem valid. Pace & Lancet (1985) published specific activities of the adenylate cyclase of the frog of about 5-10 nmoles/min/mg protein which is about ten to twenty times that found by us. Pace & Lancet, however, used the Bradford method of protein estimation (Bradford, 1976) which on these membrane preparations gives an estimate of protein concentration several times lower than methods based on the Lowry assay (Robinson, C.J., personal communication; Sklar *et al*, 1986). Correcting for the different protein assays results in a specific activity for the frog preparation of about 3-6 times that of our rat preparation. Sklar *et al* report specific activities for the frog of about 2 nmoles/min/mg protein and for the rat of about 3-4 nmoles/min/mg protein. Sklar *et al* used the Lowry protein assay so their specific activities are comparable to that of Pace & Lancet (1985). The slightly lower specific activities reported by us is probably a consequence of the different tissue preparation. Pace & Lancet (1985) estimate that 40% of the total adenylate cyclase activity of the olfactory epithelium was present in their cilia preparation but only 3% of the total protein. The slightly reduced specific activity obtained by using sonication is probably a consequence of a certain amount of epithelial tissue contaminating the cilia.

Kurihara & Koyama (1972) first reported the presence of olfactory adenylate cyclase at high specific activities (570 pmoles/min/mg protein); greater than that of even the brain. This observation was confirmed by us with olfactory adenylate cyclase activity being about 10 times that of brain membranes prepared in a similar way. Pace & Lancet (1985) reported an olfactory adenylate cyclase activity 15 times that of the brain.

In order for the activation of adenylate cyclase to be a physiologically relevant response to odour it is necessary to demonstrate that the response to odour is over a physiologically relevant range of odour concentrations. It is likely that a degree of sensitivity of the system is lost due to the inevitable contamination of the preparation with odours from the rat itself and from the compounds that would not normally have access to the receptors such as the buffer systems. It is

not yet known if the high basal activity is due to stimulation of the odorant-stimulated adenylate cyclase by the buffers and other compounds or whether it is due to the contamination with other non odour-stimulated adenylate cyclases perhaps from the underlying epithelium. Other investigators report similar stimulated to basal ratios. Pace & Lancet (1985) argued that stimulation by adenylate cyclase of odorant concentrations of μM to mM was what would one expect given that EOG experiments were usually carried out at 10^{-2} to 10^{0} of vapour saturation. These vapour saturations would result in vapour pressures of 5-0.05 mm of mercury which, given the range of water/air partition coefficients for odorants to be 1-1,000, would result in a mucus concentration of μM to mM.

The failure of citronellol and decanoic acid to stimulate the cyclase needs an explanation. Sklar *et al* (1986) investigated a series of odorants and found that only about 50% of them stimulated adenylate cyclase. They concluded that the most likely explanation was the presence of an alternative transduction mechanism and this may explain the negative results reported in this study for citronellol and decanoic acid.

The observation that calcium can inhibit both basal and stimulated adenylate cyclase activity has implications for the regulation of the system. One of the phosphodiesterase isoenzymes present in olfactory epithelium is stimulated by calcium (see chapter 3). An increase of calcium following stimulation would serve to return the system to its resting level by inhibiting the adenylate cyclase and stimulating the phosphodiesterase. The inhibition of olfactory adenylate cyclase activity by calcium was confirmed by Sklar *et al* (1986). Possible mechanisms resulting in an increase of intracellular calcium after stimulation are not known yet but a possibility is a calcium channel activated by membrane depolarisation.

Phosphoinositide breakdown forms the second messenger inositol trisphosphate which causes the release of calcium (see introduction section 1.4.1). This release of calcium has the potential to modulate the effects of cyclic AMP by interacting with the phosphodiesterase and adenylate cyclase. It is not yet known if receptor-coupled phosphoinositide breakdown is present in olfaction but if so, interactions between the two systems can occur if present in the same cell.

The stimulation of adenylate cyclase activity that was observed in the presence of GTP was minor compared to that of Pace & Lancet (1985) and Sklar *et al* (1986) who found that 10μM GTP could give rise to a doubling of activity. This difference could be due to the different membrane preparation resulting in a higher level of endogenous GTP. Another possibility is that the ATP used for the assay could have had GTP as a minor contaminant. The effects of the GTP and GDP analogues were, however, of the same order of the previous two authors. The effects of these compounds on the odorant-stimulated adenylate cyclase is good preliminary evidence for the involvement of a G protein in this stimulation. Investigations by Pace & Lancet (1986) and Anholt *et al* (1987) have demonstrated the presence of G proteins in the olfactory mucosa providing additional evidence for the involvement of a G protein in transduction (see section 1.4.3 in chapter 1).

The criteria as laid down by Sutherland (Robison *et al*, 1968) for the implication of cyclic AMP as a second messenger in a receptor cell can be adapted for olfaction as follows:

[1] The olfactory receptor cells and cilia should contain adenylate cyclase and odorants should alter intracellular cyclic AMP levels to a physiologically relevant amount.

[2] Odorants should modulate the activity of adenylate cyclase in a plasma membrane preparation from receptor cells.

[3] Inhibitors of cyclic AMP phosphodiesterases should affect the actions of odorants.

[4] Cyclic AMP or its derivatives should be able to simulate the action of odorants on the tissue.

These basic criteria are still valid today. The evidence of odorant-stimulated adenylate cyclase in the rat and frog seems to satisfy the second criterion. A note of caution must be sounded, however, in that the cilia preparation used by Pace & Lancet (1985), whilst being relatively pure, might contain contaminating plasma membranes from other cells which may contain the odorant-stimulated adenylate cyclase rather than the cilia - though this seems unlikely. The third and fourth criteria were investigated by Minor & Sakina (1972) and by Menevse *et al* (1977). Phosphodiesterase inhibitors were found to reduce the EOG response, as

did soluble derivatives of cyclic AMP; only small effects were found with soluble derivatives of cyclic GMP. These effects provide good evidence for the involvement of cyclic AMP in olfactory transduction but the drop rather than the rise in the EOG response is not what one would expect given an odorant-stimulated adenylate cyclase (see section 1.4.2 in the introduction). More studies are required to determine if this discrepancy is due to the problems of comparing disrupted tissue preparations with intact tissue or whether the alternative scheme suggested by Anholt (1987) is correct (see figure 1.6 in chapter one). The first Sutherland criterion has been investigated in this laboratory but negative results have been obtained so far, possibly due to the lack of sensitivity of the assays used (Aujla, R.A., personal communication).

One must conclude from consideration of the Sutherland criteria that much further work is required to demonstrate cyclic AMP as a second messenger. The presence of the odorant-stimulated adenylate cyclase, cyclic AMP dependent protein kinases (Heldman & Lancet, 1986), and the cyclic AMP/cyclic GMP gated ion channel (Nakamura & Gold, 1987) in cilia preparations provides good evidence, however, that this is quite likely.

3. CHAPTER THREE: OLFACTORY CYCLIC NUCLEOTIDE PHOSPHODIESTERASES.

3.1. INTRODUCTION.

3', 5'-cyclic nucleotide phosphodiesterases (PDE's) are the only known enzymes that can degrade the cyclic nucleotides generated by adenylate and guanylate cyclase. PDE was first noticed as a inactivator for the 'heat-stable factor' involved in the action of epinephrine. The 'heat-stable' factor was subsequently identified as cyclic AMP. Sutherland & Rall (1957) partially purified the inactivator, reported that the reaction product was 5'-AMP, and showed that caffeine inhibited the enzyme activity. Butcher & Sutherland (1962) further studied phosphodiesterase and discussed its possible significance for the inactivation of cyclic AMP. Since these early reports it has been demonstrated that PDE's exist in nearly all tissues studied and that they are specific towards purine nucleotide 3',5' cyclic phosphates. The availability of tritiated cyclic nucleotides in the late 1960's enabled a much more sensitive assay to be devised and this led to the discovery that the PDE's, present in many tissues, exhibited anomalous kinetics. This observation led to the discovery of multiple kinetic forms of the enzyme which, after much argument, led to the conclusion that PDE's in most tissues are present as several distinct enzyme species (isoenzymes) having different substrate specificity, distribution and kinetic properties (reviewed by Wells & Hardman, 1977; Strada & Thompson, 1978; Beavo *et al* 1982; Strada *et al* 1984; Weishaar *et al*, 1985; Erneux *et al*, 1985).

3.1.1. General properties of cyclic nucleotide phosphodiesterase.

The cyclic nucleotide phosphodiesterases (PDE's), as explained in the preceding section, are the only known enzymes that can degrade cyclic nucleotides. These cyclic nucleotides are the presumed intracellular second messengers for many tissues (see introduction to chapter 2). PDE's are obviously an important component of this system and regulation of their activity can obviously determine the intensity and duration of the cyclic nucleotide response. The activation

of at least one PDE isoenzyme by calcium allows for the linking of cyclic nucleotide mediated events and those intracellular events proceeding via calcium. It is wrong to look on PDE's as merely being the 'off-switch' for cyclic nucleotide stimulus, rather they are involved in the modulation of the signal itself to give a proper response.

The hydrolysis of cyclic nucleotides is a highly exothermic and practically irreversible reaction involving nucleophilic substitution at the phosphorus to yield a 5'-mononucleotide (Goldberg *et al*, 1984). The reaction requires a divalent cation (magnesium or manganese) for activity. In addition, sulphydryl groups seem to be important for activity; sulphydryl reagents and protease inhibitors have been shown to affect the yields and types of enzymes obtained from tissue preparations (Van Inwegen *et al*, 1976).

Most tissues seem to possess both soluble and membrane bound PDE's in varying proportions. Whilst some of the particulate activity is probably due to entrapment of activity in membrane vesicles or to loose association with the membrane, other activity cannot be washed off and probably represents an integral membrane protein. PDE from brain shows the highest proportion (up to 50%) of particulate activity, whilst most tissues only have up to 10% particulate activity. The activity of the membrane form in some tissues seems to be regulated by hormones (reviewed by Thompson *et al*, 1984).

3.1.2. Multiple enzyme forms.

3',5'-Cyclic nucleotide phosphodiesterases (PDE's) are found as multiple molecular forms or isoenzymes in most mammalian tissues (reviewed by Wells & Hardman, 1977; Strada & Thompson, 1978; Beavo *et al*, 1982; Strada *et al*, 1984; Weishaar *et al*, 1985; Erneux *et al*, 1985) and can be separated using ion-exchange chromatography and further purified to homogeneity. These isoenzymes appear to be distinct enzymes and show different substrate specificity and affinity. They can be separated from each other on the basis of size, shape, charge and other physical properties. Studies using immunologic approaches show that no antibody cross-reactivity exists between several isoenzymes, providing further evidence that they are distinct proteins rather than preparation artifacts (Hurwitz *et al*, 1984). The enzymes can be classified into

four broad types (called Type 1 to Type 4 respectively):

[1] Ca^{2+}-calmodulin stimulated PDE.

This enzyme form is distinguished by its stimulation by μM calcium ion and inhibition by EGTA and has an associated regulatory protein, calmodulin, which confers this sensitivity. Several workers have purified the soluble enzyme to apparent homogeneity by the use of calmodulin affinity columns (Strada *et al*, 1984). The enzyme seems to be dimeric, with a molecular weight of about 120,000. It is sensitive to proteolysis, which results in conversion into a fully activated form (Tucker *et al*, 1981). Calmodulin exerts its action via a calcium-dependent reversible association with the PDE. The binding of calmodulin causes an increase in the V_{max} of the enzyme but the K_m stays roughly the same (Strada *et al*, 1984). Since calmodulin is present in excess over the concentrations of PDE, the limiting factor for this PDE activity is likely to be the level of free Ca^{2+} (Sharma *et al*, 1980). This enzyme form tends to show little preference between cyclic AMP and cyclic GMP as its substrate; the K_m for cyclic GMP is usually lower than that of cyclic AMP, but the V_{max} for cyclic AMP is normally slightly greater than that for cyclic GMP. K_m values are usually about 200μM for cyclic AMP, and 10μM for cyclic GMP (Beavo *et al*, 1982).

[2] Cyclic GMP-stimulated PDE.

This enzyme form is distinguished by the ability of low concentrations of cyclic GMP to stimulate the hydrolysis of cyclic AMP, but not *vice versa*. The enzyme activity has been purified to homogeneity from bovine heart and adrenal glands (Martins *et al*, 1982). Cyclic GMP-stimulated PDE seems to have only a single polypeptide of molecular weight 105,000 and exists as a dimer. Cooperative kinetics can be observed with a Hill plot of approximate gradient 1.5, indicating positive cooperativity. Cyclic GMP seems to be acting as an allosteric activator and has two binding sites per monomer. K_m values were found by Martins *et al* (1982) for cyclic AMP and cyclic GMP to be 40μM and 10μM respectively; V_{max} values were similar for both substrates.

[3] Rhodopsin-sensitive cyclic GMP PDE.

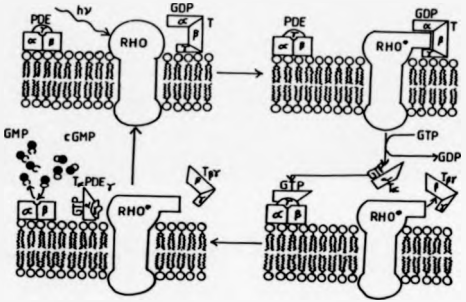
So far, this enzyme form has been shown to be present only in the rod and cone cells in the retina, which immediately distinguishes it from other isoenzymes which are found in many different cell types. It is now known that cyclic GMP is the second messenger in rod cell transduction and that the levels of this cyclic nucleotide are regulated by the coupling of the PDE to the receptor (rhodopsin) via a GTP-binding protein called transducin (Altman, 1985; Stryer 1986; Stryer 1987). The rod cell PDE was purified to homogeneity by Baehr *et al* (1979). It was found to be lightly membrane bound - washing with hypotonic buffer was sufficient to effect solubilisation. The PDE was found to consist of a core protein made up of two subunits (molecular weights 88,000 and 84,000) and an inhibitory subunit, of molecular weight 13,000. The three subunits were termed α, β and γ respectively. The PDE was found to be specific for cyclic GMP, with a K_m of 150μM for cyclic GMP and a K_m greater than 4mM for cyclic AMP. The GTP binding protein transducin has also been purified to homogeneity and characterised (Baehr *et al*, 1982). The current view of the preliminary part of visual transduction is summarised in figure 3.1.

[4] Cyclic AMP-specific PDE.

The properties of this PDE isoenzyme have been reviewed by Thompson *et al* (1984). No *in-vitro* activator for this enzyme form has yet been reported, though it seems likely that it is hormonally regulated (Thompson *et al*, 1984; Appleman *et al*, 1984). Recent work on the mechanism of insulin action suggests that a second messenger, inositol phosphate-glycan (IPG), can activate cyclic AMP PDE and inhibit adenylate cyclase (Espinal, 1987; Saltiel & Cuatrecasas, 1986). The soluble enzyme from dog kidney has been purified to homogeneity and characterised by Thompson *et al* (1979b). Cyclic AMP-specific PDE appears similar in its properties to both soluble and particulate cyclic AMP PDE's isolated from various other tissues (Thompson *et al*, 1984). This isoenzyme is distinguished from other PDE's by its high affinity and specificity for cyclic AMP, with K_m values for cyclic AMP usually of the order of fractional μM and K_m values for cyclic GMP of several hundred μM. The enzyme, purified by Thompson *et al* (1979b), was shown to have a molecular weight of about 60,000. Non-Michaelis-Menton kinetics are often seen for this enzyme form indicating the

Figure 3.1. Proposed transduction mechanism for the light activated conductance in rod cells of the retina.

The cycle begins with the absorption of a photon by rhodopsin (upper left). Activated rhodopsin then interacts with transducin (T), as shown at the upper right. That interaction causes GTP to replace GDP in the alpha subunit of transducin and causes the alpha unit to split off from the beta-gamma part of the enzyme (lower right). Transducin relieves the inhibition of the inactive phosphodiesterase (PDE), perhaps by carrying away its gamma unit. The activated phosphodiesterase begins cleaving many molecules of cyclic GMP (lower left). Before long a built-in timer in the alpha subunit of transducin cleaves GTP to GDP. The alpha subunit rejoins the beta-gamma unit; the phosphodiesterase is also reassembled. At the same time rhodopsin is inactivated and then regenerated in its preactivation form. Taken from Stryer (1987)



probable presence of negative cooperativity; Thompson *et al* (1984) demonstrated the presence of both high and low affinity binding sites for cyclic AMP.

The presence of PDE isoenzymes, which can vary from one organ to another, and the identification of numerous diverse PDE inhibitors (Weishaar *et al*, 1985) has led to increasing interest from the pharmaceutical industry as to PDE's being possible drug targets. The 'classic' PDE inhibitors, 3-isobutyl-1-methylxanthine and theophylline, are generally non-selective for the isoenzymes. A very large number of inhibitors, which are broadly selective for the PDE isoenzymes, have been identified over the last few years. These compounds will probably be of use as positive inotropic agents for the treatment of congestive heart failure; as mediator-release inhibitors for the treatment of asthma and related pulmonary disorders; and as platelet aggregation inhibitors for use in the treatment and prevention of ischemia (Weishaar *et al*, 1985).

3.1.3. Cyclic nucleotide phosphodiesterases in olfaction.

Recent evidence has suggested that cyclic AMP serves as a second messenger in olfaction (see chapters 1 and 2). The adenylate cyclase enzyme is present in relatively high concentrations in olfactory mucosa and has been shown to be stimulated by odorants *in vitro*.

The Sutherland criteria for cyclic AMP as a second messenger requires the presence of a corresponding cyclic nucleotide PDE. It has been shown (Menevse *et al*, 1977; Minor & Sakina, 1972) that membrane permeable PDE inhibitors can inhibit the production of the electro-olfactogram (EOG) in the frog, implicating the importance of cyclic nucleotides. This EOG is a surface potential, produced in response to an odour, and is caused by the flow of the generator current of the olfactory primary cells (see section 1.2.1 in chapter 1). The EOG is an indicator of one of the earliest electrical events in odour transduction. This inhibition of the EOG was observed with cyclic AMP analogues, but not with cyclic GMP analogues. If cyclic AMP was the second messenger in olfaction one would expect an increase in the EOG and not a decrease as observed - this paradox is discussed further in chapter 1 (section 1.4.2).

The presence of cyclic AMP PDE's in olfactory mucosa has been shown by polyacrylamide gel electrophoresis, and the changes in tissue-specific isoenzyme patterns produced on

denervation have been identified (Margolis, 1977). There are no data on the levels of the PDE enzymes, however, and no attempt to show the presence of cyclic GMP PDE.

3.1.4. Aims.

Apart from the study of Margolis (1977) on the isoenzyme patterns during denervation, and the studies by Menevse *et al* (1977) and Minor & Sakina (1972) on the effect on EOG's using membrane permeable PDE inhibitors, there are no biochemical studies of the PDE enzymes found in olfactory tissue. The odorant-stimulated adenylate cyclase requires the presence of a PDE in sufficient amount to return the levels of cyclic AMP to basal levels. The high activity of adenylate cyclase in olfactory tissues will require appropriately high levels of PDE.

The inability of all odorants to stimulate olfactory adenylate cyclase strongly suggests the presence of an alternative transduction mechanism. The demonstration of a unique receptor-coupled PDE in another sensory system, namely visual transduction (Stryer, 1986; Stryer, 1987), suggests the possibility of a similar system occurring in olfaction.

It was therefore decided to undertake a study of the PDE's in olfactory tissue, in order to determine whether they resemble those found in the majority of tissues in the body, or whether they resemble the PDE found in the retina.

3.2. EXPERIMENTAL.

3.2.1. Materials.

[8- ^{3}H] cyclic AMP (23.6Ci/mmole), [8- ^{3}H] cyclic GMP (15Ci/mmole), [U- ^{14}C] adenosine (538mCi/mmole) and [U- ^{14}C] guanosine (502mCi/mmole) were obtained from Amersham International (Bucks., U.K.). The tritiated cyclic nucleotides were purified before use as described below. Snake venom (*Ophiophagus Hannah*) was obtained from Sigma Chemical Company (Poole, Dorset, U.K.). Cyclic AMP (99%) and cyclic GMP (98%) were also obtained from Sigma and were not further purified. Dowex 1X2-400 was obtained from Sigma and prepared for use by washing successively with 0.5M HCl, H_2O, 0.5M NaOH, H_2O, 0.5M HCl, and H_2O. The resin

was then washed repeatedly with water until pH 5 was reached. DE-52 was obtained from Whatman LabSales Ltd (Maidstone, Kent, U.K.). DEAE Sephacel, polyethyleneimine thin layer chromatography plates and bovine brain calmodulin were obtained from Sigma. All other biochemicals and chemicals were obtained from Sigma, BDH Chemicals (Poole, Dorset, U.K.) and Aldrich Chemical Company Ltd (Gillingham, Dorset, U.K.) and were the highest purity commercially obtainable. All buffers were made up in distilled water passed through a Millipore 'Milli-Q' system (18 Megohms/cm).

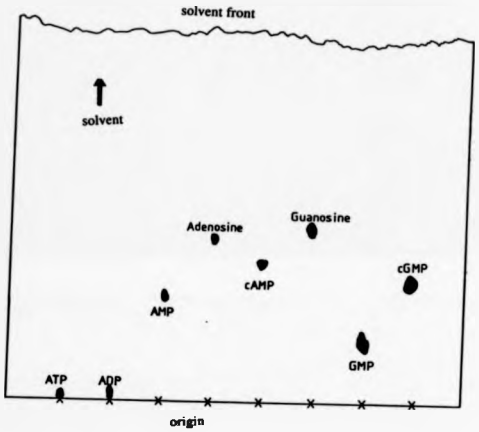
3.2.2. Purification of tritiated cyclic nucleotides.

It was found that purification of tritiated cyclic nucleotides was necessary to reduce assay blanks to below 5% of added radiolabel. The purification, presumably, works by removing uncharged contaminants which would not be removed by the separation procedure in the assay. The method of Bohme & Schultz (1974) was adapted for the purification as described below.

Polyethyleneimine (PEI) cellulose thin layer chromatography plates were used. The plates were scored in a line 1cm from the edges to help even running and then approximately 20μCi of ligand was loaded in a tight spot. 10nmoles of unlabelled cyclic nucleotide was loaded on either side of the ligand, to act as a marker. The plates were developed using 1M ammonium acetate buffer at pH 5.4 for approximately three hours. When developed, the ammonium acetate was removed by heating the plate in an oven at 60°C for the minimum time necessary to dry the plate. When dry, the inbuilt UV marker of the plate allowed visualisation of the marker spots when illuminated at 254nm with a UV lamp. The presumed location of the radiolabel was then cut out and suspended in 2mls assay buffer. The tube was vortexed vigorously, left for 15 minutes and then centrifuged. The purified ligand was aliquoted out and stored at -20°C. Recovery of radiolabel from the plate was usually about 75%. Assay blanks remained stable for a few weeks subsequent to the separation. Figure 3.2 shows the separation achieved by this system for guanosine, adenosine and a variety of nucleotide derivatives.

Figure 3.2. Separation of purine nucleotides and nucleosides using PEI thin layer chromatography.

The nucleotides and nucleosides were loaded as a tight spot of 10nmoles. The plates were developed using 1M ammonium acetate pH 5.4. When the solvent had reached near to the top, the plates were removed and dried in an oven. The fluorescent indicator in the plates allowed visualisation of the spots when viewed at 254nm.



3.2.3. Tissue preparation.

[1]. Preparation of rat tissue.

Male Wistar rats, approximately 300g in weight were used. They were allowed food and water *ad libitum* prior to the experiment. The rats were stunned and killed by cervical dislocation. The olfactory turbinates, septum and the covering membrane were removed, after sagittal sectioning of the head using a knife, and suspended in ice-cold assay buffer (40mM Tris.Cl, 10mM $MgCl_2$, pH 8.0). The turbinates were washed once with the assay buffer to remove blood and then sonicated in a MSE 100 W sonicator, fitted with an exponentially tapered probe of 3 mm tip diameter, for three bursts of five seconds (12 microns peak to peak) in 1ml of ice-cold assay buffer per set of turbinates. This relatively light sonication left the cartilage structure largely intact and presumably just removed the cilia and possibly the outer epithelial layers. The homogenate was then spun at 500xg for 15 minutes to pellet the cartilage and other tissue fragments, and the supernatant used for routine phosphodiesterase assays. The soluble enzyme was obtained by spinning the tissue homogenate at 100,000xg for one hour in an ultracentrifuge. When required, the pellet from the 1000,000xg spin was resuspended in assay buffer by sonication. The soluble enzyme was also used for the column chromatography with the difference that the tissue was prepared in the buffer used for the chromatography.

Whole rat brain was removed and chopped up before sonication, then prepared in the same way as the olfactory tissue, when required, as a control tissue.

Protein concentrations were estimated, by using the Hartree variation of the Lowry method (Hartree, 1972), using bovine serum albumin fraction V (96-99%) from Sigma as the standard. Approximately 4mg of soluble protein were obtained from each rat; approximately the same amount of protein was found in the particulate fraction.

[2]. Preparation of sheep tissue

Due to the small amount of tissue obtained from a rat, at least 25 rats would have been required for a adequate separation of the phosphodiesterases in olfactory tissue. The ethical considerations and expense of handling this number of animals led to a search for other experimental animals. It

was decided that sheep would be suitable as they were being used in this laboratory for studies on cytochrome P-450. Sheep heads were obtained from the local abattoir immediately after death and transported back to the laboratory. The location of the olfactory epithelium, deep in the head, meant that it would have been pointless to transport the heads in ice, since the delay between death and start of dissection was only 15-30 minutes. The heads were skinned using a Stanley knife and then sagittally sectioned using a 12 inch hacksaw fitted with a coarse blade (18 teeth/inch). The septum was cut and prised away and the olfactory region (visible by its darker pigmentation than the surrounding tissue) was removed, using scissors to cut through the turbinates. Approximately 5g of tissue was obtained from each sheep head. The tissue was homogenised using an 'Ultraturrax' (rotating blade type) homogeniser in 3 volumes of column separation buffer (40mM Tris.Cl, 1mM DTT, 0.25mM PMSF, 5mM Glucose, 30% ethylene glycol, pH 7.5) in several short bursts until most of the tissue was fragmented. Centrifugation at 32,000xg for 1 hour using an SS-34 rotor yielded the soluble preparation used in the studies. When required the pellet was resuspended in buffer using the Ultraturrax and centrifuged at 500xg for 15 minutes to remove large tissue fragments and cartilage.

The supernatant from this preparation gave about 10mg/ml protein using the Hartree protein assay (Hartree, 1972) with BSA as the standard. The pellet from the preparation, when resuspended in an equal volume to the supernatant, also gave about 10mg/ml protein. One sheep, therefore, gave approximately 50 times the total protein of one rat. The specific activity of the sheep soluble phosphodiesterases was about half that of the rat so each sheep yielded 25 rats worth of phosphodiesterase. The slightly reduced specific activity was probably due to the homogenisation of the total turbinate area of the sheep which would contain an amount of structural protein that would not have been included with the light sonication used in the rat preparation. The large tapered sonication probes did not result in satisfactory homogenisation of sheep turbinates even on full power.

3.2.4. Phosphodiesterase assay.

The assay method was the batch method of Thompson *et al* (1974, 1979a) which converts

the mononucleotides formed during the assay to uncharged nucleosides by incubating the reaction products with King Cobra snake venom (*Ophiophagus Hannah*). The venom contains a suitable nucleotidase but no other enzymes which could cause interference with the assay. The uncharged nucleosides can then be easily separated from the charged substrate by use of an anion exchanger, added directly to the assay tube and then centrifuged. The buffer used for the assay comprised 40mM Tris.Cl, 10mM $MgCl_2$, pH 8.0. Mercaptoethanol, DTT or PMSF were not used at first as these protective agents are odorants - no problems were noted due to this omission. For subsequent assays on the sheep, 1mM DTT was added, to reduce any disulphide bonds formed by oxidation (Cleland, 1964). The reaction was linear with the above conditions for at least one hour. 12ml disposable plastic assay tubes were used, and the assay mixture consisted of the following;

[1] 100µl of ^3H cyclic AMP or cyclic GMP (=20,000cpm) and ^{14}C adenosine or guanosine (=5,000cpm) in assay buffer. The concentration of radiolabelled nucleotide is about 2nM and can be neglected.

[2] 100µl of unlabelled cyclic AMP or cyclic GMP, to the desired concentration of nucleotide. In addition if calcium, calmodulin, EGTA or odorants were required they were added at this point. Unlabelled cyclic nucleotides were weighed out roughly and then the exact concentrations were calculated by using the extinction coefficients at 259nm and pH7.0 (εmM cyclic AMP=14.65, εmM cyclic GMP=13.7) as given by Thomson *et al* (1974). The solutions were then aliquoted out and stored at -20°C until required.

[3] 200µl of diluted enzyme in assay buffer containing 1mg/ml of BSA was added to start the reaction. The enzyme concentration was adjusted so that the turnover of substrate during the 30 minute incubation period did not exceed 20%.

The tubes were incubated at 30°C for exactly 30 minutes and the reaction terminated by boiling the tubes for exactly one minute. Nucleotide monophosphates formed during the incubation were then cleaved to their corresponding nucleosides by the addition of 100µl of 1mg/ml snake venom in water, followed by a 10 minute incubation at 30°C. The tubes were then placed on ice and 1ml of a slurry consisting of 1 part Dowex-1X2-400 anion exchange resin and 4

parts methanol was added to absorb any charged, unreacted nucleotides. Resin was added to the methanol shortly before use and then gently stirred. The total aqueous volume of the assay mixture at this point was 1.4ml; the ion-exchanger was found to take up the remaining 0.1ml. The tubes were vortexed and incubated on ice for 15 minutes, after which they were vortexed again and spun at 500xg for 10 minutes in an MSE Mistral clinical centrifuge to pellet the resin. 0.5ml aliquots of the supernatant were taken into 'mini' plastic scintillation vials and then 5ml of scintillation fluid was added (LKB Optiphase-'safe').

The tubes were then shaken and counted in either a Packard 'Tri-Carb' or 'Prias' scintillation counter. Quench curves were set up in order to correct for quench and overspill from the ^{14}C to the ^{3}H channel, using the external quench standard option in the machines. Assay blanks followed every duplicate or triplicate determination and were composed of 200µl of assay buffer with 1mg/ml BSA instead of enzyme. No difference was found between these blanks and boiled enzyme fractions.

Assay blanks were usually between 2% and 5% of added tritium label. Cyclic GMP blanks were usually about half that of cyclic AMP blanks, which were more dependent on the care taken during purification of the radiolabel and the time that the purified nucleotide had been stored. Increased blank values would either result from tritium exchange of the radiolabelled nucleotide with water or breakdown of the tritiated cyclic nucleotide on storage. Blanks could be reduced to about 1% by using water instead of methanol (Thompson *et al*, 1974) but this led to recovery of product dropping to about 40%.

Recovery of product was corrected by following the fate of ^{14}C labelled nucleosides and was usually about 90%, this parameter was calculated by use of the following expression;

$$Recovery\ (R) = \frac{^{14}C\ counts - background}{(^{14}C\ total \times 0.5/1.4) - background}$$

Using the tritium counts and the recovery of the nucleosides as calculated above, the fraction of the substrate that had been metabolised could then be calculated;

$$Turnover\ (T.O) = \frac{^{3}H\ counts - blank}{(^{3}H\ total \times 0.5/1.4 \times R) - blank}$$

The activity of the enzyme in units of pmoles/min/µl could then be calculated using the following expression;

Activity = pmoles substrate per assay × T.O/30/µl enzyme used per assay

The specific activity of nmoles/min/mg protein can then be calculated by dividing the above activity by the concentration of protein in mg.

3.2.5. Column Separation of olfactory Phosphodiesterases.

The soluble phosphodiesterases (PDE's) were separated using ion-exchange chromatography on DEAE Sephacel or Whatman DE-52. Two slightly different techniques were used depending on whether rat or sheep homogenates were to be used.

[1] Separation of rat olfactory phosphodiesterases.

Due to the small amount of tissue obtained from a rat the separation technique of Strada *et al* (1984) was scaled down accordingly. The tissue from four rats was prepared in 0.015M sodium acetate buffer containing 1mM EGTA at pH 6.4. No protective agents were used as it was intended to attempt odour modulation studies on any separated isoenzymes. The tissue was then applied to a Pharmacia K9 column (9x150mm, 9.6ml bed volume) containing DEAE Sephacel equilibrated with the acetate buffer. Two bed volumes of the acetate buffer were passed through the column and then the PDE's were eluted using a 48ml linear gradient. This gradient was generated using a Pharmacia 'P-3' three channel peristaltic pump, going up to 1.5M acetate in the acetate buffer, with a flow rate of 6.4ml per hour. Linear gradients were generated according to Lakshmann & Lieberman (1954), which consisted of pumping the limit buffer into a stirred reservoir of starting buffer at half the rate pumped out onto the column. The concentration of sodium acetate in the fractions was not determined due to the scarcity of sample. The assumption of a linear gradient seems justified as experiments on the sheep using a much cheaper pump gave a good linear gradient.

1ml fractions were taken and 50µl of these fractions were taken for the PDE assay at 100µM cyclic AMP. The effect of sodium acetate on the phosphodiesterase assay blanks was corrected by using boiled enzyme fractions.

[2]. Column separation of sheep olfactory phosphodiesterases.

The sheep phosphodiesterases were separated using ion-exchange chromatography on either Whatman DE-52 or DEAE Sephacel using the method of Thompson *et al* (1979a). The large amount of tissue from the sheep meant that the separation procedure did not have to be scaled down. Due to the lack of specific odour effects on phosphodiesterase activity and the lengthy column separation, it was decided to use DTT and PMSF in the homogenisation buffer and separation buffer. DTT serves to maintain thiols in their reduced state and has the advantage over mercaptoethanol of having a much reduced odour and increased stability to air oxidation (Cleland, 1964). PMSF was used to inhibit proteolysis by sulphonating serine residues at the active site of proteases (Gold, 1967) and was made up immediately before use as a concentrated 250mM solution in ethanol, and added at the start of homogenisation. Sulphydryl reagents and protease inhibitors have been shown to alter the yields and isoenzymes obtained by ion-exchange of PDE's (Van Inwegen *et al*, 1976). Thompson *et al* (1979a) reported that the inclusion of 30% ethylene glycol and 5mM glucose in buffers enhanced yields and allowed separated phosphodiesterases to be stored at -20°C without freezing for several months with enzyme stability. I found that ethylene glycol and glucose had no effects on enzyme activity.

The tissue from two sheep was homogenised according to section (3.2.3) in 30 mls of homogenisation buffer (40mM Tris.Cl, 1mM DTT, 0.25mM PMSF, 5mM glucose, 30% ethylene glycol, pH 7.5). The soluble fraction (25ml) was then applied to a 1.5x30cm column (Wright Scientific Ltd) containing either DEAE Sephacel or Whatman DE-52, pre-equilibrated with starting buffer (70mM sodium acetate, 1mM DTT, 5mM glucose, 0.25mM PMSF, 30% ethylene glycol, pH6.5). The column was then washed with 100mls of starting buffer. The phosphodiesterases were then eluted using 400 ml linear gradient of 70mM to 1000mM sodium acetate in starting buffer. A modified three channel Schuco pump was used fitted with 1.0mm bore size silicone rubber sleeving (Cynflex, Nottingham, U.K.). A flow rate of 40ml/hour was used. The linear gradient as generated according to Lakshmann & Lieberman (1954). Fractions of 5ml were taken and stored at -20°C until used. The sodium acetate concentration in the fractions was estimated using a Metrohm Herisau Konduktometer E527 conductivity bridge, fitted with a

Philips PW9510 1cm conductivity cell and calibrated by using sodium acetate standards. High concentrations of sodium acetate affected the blanks in the phosphodiesterase assay. Correction for the alteration of blank values was carried out, using either boiled fractions or a standard calibration curve of blank value with sodium acetate concentration. No differences were noted between the two methods.

3.3. RESULTS.

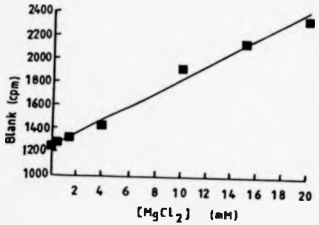
3.3.1. General properties of olfactory phosphodiesterases.

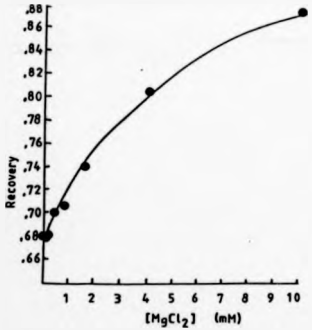
Large amounts of phosphodiesterase (PDE) were found in both sheep and rat olfactory tissue. The rat enzyme was found to produce a rate of breakdown of cyclic AMP that was linear with time for up to one hour. The presence of sulphydryl reagents would not, therefore, seem to be necessary. The reaction rate was also found to be linear with respect to changing enzyme concentration over a ten-fold range, suggesting that the PDE enzyme is stable when diluted in the 1mg/ml BSA containing buffer.

Magnesium ion, as expected, was found to be necessary for rat cyclic AMP PDE enzyme activity. The metal-ion chelating agent EDTA, at 1mM in the assay, was sufficient to abolish all enzyme activity. The addition of 100μM magnesium chloride to the assay resulted in a maximal enzyme activity which was double that obtained with no additional magnesium ions added. This observation suggests that magnesium was a contaminant of either the tissue preparation or of the substrate. The blank value for the assay was found to vary with magnesium ion concentration as shown in figure 3.3. A roughly linear relationship between blank and magnesium ion concentration was obtained, with a doubling of blank value for 20mM additional magnesium ions. This observation was noted by Thompson *et al* (1979a). The recovery of the assay was also found to vary with magnesium ion concentration, as shown in figure 3.3, from about 0.68 at no additional magnesium to 0.87 at 10mM magnesium ion. The recommended concentration of magnesium in the assay is about 5mM (Thompson *et al*, 1979) which gives a maximal enzyme activity, good recovery and a blank assay value that is not too excessive. It was found that the

Figure 3.3. Effect of Magnesium ion on the PDE assay blank and recovery values.

The PDE assay, as described in the methods section, was modified by the addition of varying amounts of magnesium chloride; 100μM cyclic AMP was the substrate. The blank values are given as cpm ^3H. Recoveries of adenosine were estimated by following ^{14}C adenosine as described in the methods section with about 10% substate conversion during the assay. All points are the average of duplicate determinations from a single experiment.





sodium acetate used for the elution of the PDE's during the column chromatography also had an effect on the reaction blank. This effect was not as great as that for magnesium chloride; an increase in sodium acetate concentration, from zero to 300mM, resulted in an increase of the blank of 50%.

Kinetic parameters were estimated for the crude rat olfactory homogenate, and representative double reciprocal plots for the cyclic AMP and cyclic GMP olfactory enzymes are presented in figure 3.4. The typical non-linear reciprocal plots often seen for crude tissue preparations of phosphodiesterases (PDE's) are seen for olfactory tissue over a substrate range of 0.8-100μM. The non-linear reciprocal plots suggest the presence of isoenzymes, cooperative kinetics, or an artifact due to vesicles present in the tissue preparation.

The graph seems to have two linear portions which can be extrapolated to yield apparent K_m values for the high and low affinity enzymes. These values are given, along with the apparent V_{max} values in table 3.1. There is approximately two to three times as much cyclic AMP PDE than cyclic GMP PDE activity as measured at saturating substrate concentrations. At substrate concentrations of 2μM the activities become comparable due to the higher affinity of the PDE for cyclic GMP.

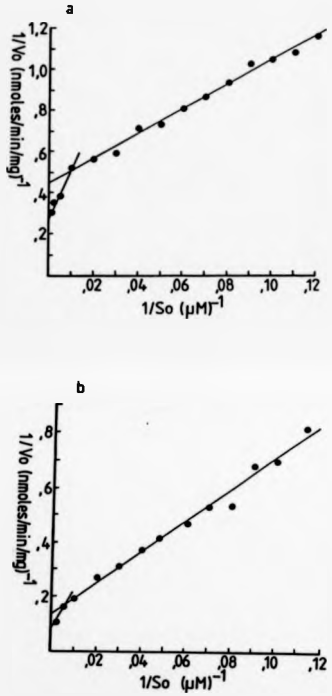
Rat brain homogenate, prepared in the same way as the olfactory homogenate, showed V_{max} values for the hydrolysis of cyclic AMP and cyclic GMP of 33 nmoles/min/mg protein and 29 nmoles/min/mg protein respectively (single preparation). These activities are approximately five to ten times the activity of the olfactory PDE enzyme. The brain is usually considered to have the highest PDE activity (Strada *et al*, 1984).

Crude sheep olfactory homogenate also had high levels of PDE activity. There was about half the specific activity of rat cyclic AMP PDE but equivalent values of cyclic GMP PDE (about 3nmoles/min/mg protein for both).

Both cyclic AMP and cyclic GMP PDE activity were present in the soluble and particulate fractions of rat olfactory homogenate after ultracentrifugation. Results from triplicate determinations with three tissue preparations yielded 78 ± 3% soluble cyclic AMP PDE activity

Figure 3.4. Double reciprocal plots of cyclic AMP and cyclic GMP PDE activity ('a' and 'b' respectively) in crude sonicates of rat olfactory epithelium.

Two typical results are shown with each point being the mean of duplicate determinations. Lines were fitted using linear regression analysis. Kinetic parameters of rat olfactory PDE's are given in table 3.1.



	cyclic AMP PDE	cyclic GMP PDE
K_m1 (micromolar)	2.9 ± 0.6	1.6 ± 0.4
K_m2 (micromolar)	19 ± 3.0	15 ± 4.0
$V_{max}1$ (nmoles/min/mg)	3.4 ± 0.4	2.1 ± 0.4
$V_{max}2$ (nmoles/min/mg)	6.3 ± 0.9	3.4 ± 0.2

Table 3.1. Kinetic parameters of cyclic AMP and cyclic GMP phosphodiesterases from rat olfactory tissue.

Kinetic parameters of olfactory phosphodiesterase activities in crude olfactory sonicates. The apparent K_m and V_{max} values were obtained from double reciprocal plots using least-squares linear regression analysis. Units are as given in the table. Values shown are the means (± S.E.M.) from three experiments using different animals.

and 72 ± 2% soluble cyclic GMP PDE activity (means ± S.E.M.). The activity in the pellet could represent a genuine membrane-bound enzyme, or could result from soluble PDE trapped in membrane vesicles. The hypotonic buffer used for the sonication would have resulted in the solubilisation of a peripherally membrane-bound PDE, as found by Baehr *et al* (1979) in their purification of cyclic GMP PDE from rod outer segments. Similar levels of soluble cyclic AMP and cyclic GMP PDE's were found in sheep olfactory homogenates; 70% and 73% respectively from a single determination.

Both rat olfactory PDE's were stable to freeze-thaw cycles when stored at -20 °C, the first thaw usually resulting in about 25% more activity than the fresh tissue. This could be have been due to the loss of a regulatory protein or the rupture of closed vesicles on freezing and thawing. Subsequent freeze-thaw cycles (up to three or four times) resulted in a constant activity of enzyme. The effect of freeze-thaw cycles on the individual isoenzymes would have to be

determined to assess the effects that freezing has on PDE activity. Sheep olfactory PDE's were stable when stored at -20 °C in the 30% ethylene glycol containing buffer and showed no change in activity after storage.

Both rat cyclic nucleotide PDE's were calcium-sensitive (table 3.2) but considerably less so than the corresponding brain tissue. The sensitivity seems to be in the low µM range, as 20µM additional calcium resulted in maximal stimulation. The concentration of calcium in the tissue homogenate is not known. The presence of a portion of the enzyme activity that is inhibited by the calcium chelating agent EGTA is usually taken to indicate the presence of the Type 1 PDE which, as discussed in the introduction, has an associated calmodulin sub-unit that confers calcium sensitivity. Sheep olfactory homogenate showed slightly less calcium stimulation for cyclic AMP PDE of 144 ± 7% and for cyclic GMP PDE of 128 ± 5% (means ± S.E.M. of three experiments). The addition of extra bovine brain calmodulin to the assay did not result in any increase of enzyme activity for either rat or sheep olfactory PDE's, suggesting either that calmodulin is present in excess in the tissue homogenate or that the PDE isoenzyme has a very tightly bound calmodulin sub-unit.

	cyclic AMP PDE	cyclic GMP PDE
Brain	262 ± 5%	274 ± 15%
Olfactory	151 ± 15%	136 ± 6%

Table 3.2. The calcium stimulation of rat PDE activity of both brain and olfactory homogenates.

The crude rat olfactory sonicate was incubated with 100µM cyclic AMP or cyclic GMP in the presence of 100µM EGTA or 1mM additional calcium. Values presented are the percentage increase of activity with calcium present. Values shown are the mean (± S.E.M.) for three replications on three different animals.

3.3.2. Effects of odorants on phosphodiesterase activity.

Table 3.3 lists the effect of six well known odorants on the crude rat olfactory PDE activities at 2μM substrate, compared to brain PDE prepared in the same way. The results suggest that there are no dramatic differences between the response of brain and olfactory PDE's towards the odorants. The observed effects are probably due to the lipophilic odorants binding non-specifically to the enzyme and disrupting its function. The pyrazine was a weak competitive inhibitor as expected from its structural similarities to other PDE inhibitors (Weishaar *et al*, 1985) and gave an approximate K_i of 1 mM (single determination).

	Cyclic AMP		Cyclic GMP	
	Brain	Olfactory	Brain	Olfactory
Carvone	59 ± 3%	71 ± 2%	84 ± 3%	87 ± 6%
Cineole	99 ± 6%	107 ± 2%	90 ± 1%	104 ± 5%
iso-Valeric acid	108 ± 3%	99 ± 3%	90 ± 6%	100 ± 3%
2-Isobutyl-3-methoxy pyrazine	55 ± 3%	59 ± 1%	57 ± 5%	69 ± 1%
Trimethylamine	95 ± 5%	99 ± 5%	110 ± 9%	98 ± 5%
Acetophenone	82 ± 2%	84 ± 5%	91 ± 9%	86 ± 3%

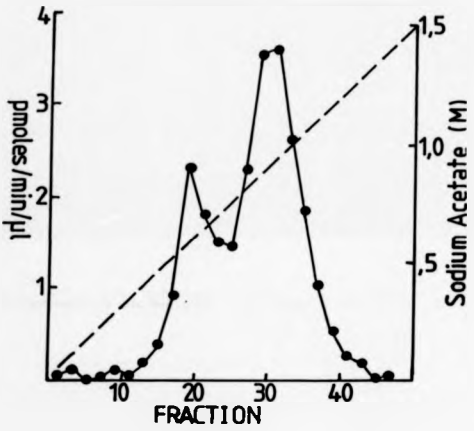
Table 3.3. Effect of odorants on brain and olfactory PDE activity from the rat.
The effect of odorants was assayed on both cyclic AMP and cyclic GMP PDE at 2μM substrate. All odours are at 4mM in the assay. Values are the mean (± S.E.M.) percentage deviation from the basal value for triplicate determinations on a single preparation.

3.3.3. Resolution of olfactory phosphodiesterase isoenzymes.

Ion-exchange chromatography of the soluble PDE's of the rat, assayed at 100μM cyclic AMP, resolved two peaks (figure 3.5). Neither of the two fractions were stimulated by calcium in the absence of added calmodulin. Table 3.4 shows the effect of calcium on the two peaks when in the presence of 80ng of bovine brain calmodulin. The first peak was stimulated by 200% whereas

Figure 3.5. Separation of soluble PDE isoenzymes from rat olfactory epithelium.

The soluble rat homogenate was applied to a 9.6ml column of DEAE Sephacel equilibrated with 15mM sodium acetate, 1mM EGTA pH 6.4. Activities were eluted at 6.4ml per hour with a 48ml linear gradient of 15mM to 1.5M sodium acetate. 1ml fractions were collected and the PDE activities of 50µl aliquots were measured at 100µM cyclic AMP. Activities were corrected for the increase of assay blank with increasing ionic strength. Values shown are the mean of duplicate determinations. The estimated sodium acetate concentration of the fractions is given in the figure.



the second was unaffected. The results suggest that the calcium stimulation observed in tissue homogenates results from the presence of a PDE isoenzyme containing a bound calmodulin subunit; the ion-exchange chromatography results in the loss of this subunit. The calcium sensitivity can be restored by the addition of calmodulin to the assay. The existence of this calcium/calmodulin regulated PDE has been shown for a variety of other tissues (Erneux *et al*, 1985) and it is known that DEAE cellulose chromatography in the presence of EGTA can cause separation of the calmodulin. Binding of the calmodulin to at least one of the Type 1 PDE isoenzymes is dependent on calcium ions being present (Strada *et al*, 1984).

Fraction	Basal	+ Calcium/calmodulin
20	2.13 ± 0.5	4.05 ± 0.11
31	3.23 ± 0.03	3.13 ± 0.04

Table 3.4. The calcium dependence of the two separated olfactory phosphodiesterases.
The fractions corresponding to the two peaks were assayed at 100μM cyclic AMP. The fractions were assayed at 100μM EGTA, to determine basal rate, or with 1mM calcium chloride and 80ng of bovine brain calmodulin to determine the stimulated activity. Activities are presented as pmoles/min/μl of fraction. The values are the mean (± S.E.M.) from a triplicate determination.

The small amount of tissue obtained from the rat meant that assaying the column fractions and assessing the calcium/calmodulin stimulation used up most of the relevant fractions. In order to attempt a full characterisation of the isoenzymes in olfactory tissue a separation using the homogenate from two sheep (the equivalent of 50 rats) was carried out. Using sheep had the advantage that the separation procedure of Thompson *et al* (1979a) did not have to be scaled down. The use of ethylene glycol and glucose in the buffers used for the separation would have improved the yield and allowed the storage of any fractions at -20°C without loss of enzyme-sensitivity to activators (Thompson *et al*, 1979a). The lack of any odour effects on the rat PDE allowed the inclusion of DTT to reverse the effects of any air oxidation and PMSF to inhibit proteolysis. The additional compounds used in the experiment resulted in no difference in PDE

activity when assayed under standard conditions.

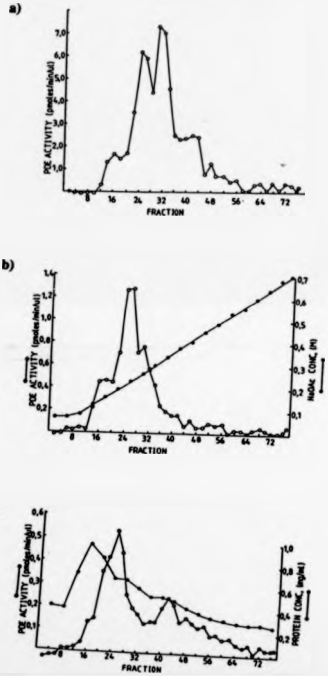
The activity of the fractions from the ion-exchange separation of the soluble PDE's of the sheep are shown in figure 3.6. The fractions were assayed under three substrate conditions as per Thompson *et al* (1979a). The three elution profiles show the presence of three peaks corresponding to fractions 24, 30 and 42. There is a possible fourth peak present as a shoulder to the first peak in all three elution profiles, which corresponds to fraction 18.

The differences between the rat and sheep elution profiles could be due to the experimental animals having different isoenzymes present. The different homogenisation methods used on the sheep could, however, have released PDE isoenzymes deeper in the epithelium. The effect of the two different ion-exchangers and buffers cannot be neglected; repeating the separation of the sheep isoenzymes but using DEAE Sephacel rather than DE-52 led both to the peaks being eluted at much higher salt concentration, and to considerable broadening of the peaks. Comparisons of the elution profiles obtained here show broad similarities with those reported for many other tissues (Wells & Hardman, 1977; Strada & Thompson, 1978; Beavo *et al*, 1982; Erneux *et al*, 1985). It is usually found that the three different substrate concentrations used to assay the peaks complement each other. However, I found that assaying the fractions at 100μM cyclic AMP was sufficient to lead to the identification of all of the olfactory PDE isoenzymes.

The extra volume and activity of fractions resulting from the sheep separation allowed a characterisation of the separated isoenzymes. The usual strategy reported in the literature is to attempt to identify calcium/calmodulin and cyclic GMP stimulated forms. The effect of EGTA, calcium and calmodulin on the four fractions at 100μM cyclic AMP is shown in table 3.5. The results clearly indicate that the second peak is a calcium/calmodulin regulated form (the so-called Type 1 isoenzyme). The slight stimulations shown for the other peaks are probably a consequence of slight contamination of the peaks with the Type 1 PDE. Assaying the Type 1 PDE at 100μM cyclic GMP showed a slightly reduced stimulation with calcium and calmodulin of 185 ± 1% (mean of triplicate experiments ± S.E.M.). This slight reduction in calcium ion stimulation for cyclic GMP hydrolysis was also reflected for the original homogenate which showed a maximal

Figure 3.6. Separation of soluble PDE isoenzymes from sheep olfactory epithelium.

Fractions from DE-52 chromatography of soluble sheep homogenate were assayed for PDE activity with 100μM cyclic AMP, 1μM cyclic AMP and 1μM cyclic GMP (labelled 'a', 'b' and 'c' respectively). The sheep homogenate was loaded onto a 1.5x30cm column of DE-52 equilibrated with buffer (70mM sodium acetate, 1mM DTT, 0.25mM PMSF, 5mM glucose, 30% ethylene glycol, pH 6.5). Activities were eluted at 40ml per hour with a 400ml linear gradient of 70mM to 1.0M sodium acetate. Protein concentrations and sodium acetate concentrations were estimated as described in the methods. 5ml fractions were collected and assayed for PDE activity. Values shown are the mean of duplicate determinations after correction for the rise in assay blank with sodium acetate concentration.



calcium ion stimulation of 128 ± 5% (mean of three experiments ± S.E.M.).

The results contrast with those of the rat, in that the ion-exchange procedure did not seem to cause separation of the calmodulin sub-unit to any great extent. This difference may have been due to the use of EGTA in the buffers which were used for the ion-exchange separation of rat olfactory PDE's. However, it has been shown that ion-exchange chromatography in the absence of EGTA can still cause loss of the calmodulin subunit (Strada *et al*, 1984). It is possible that sheep olfactory tissue has a Type 1 PDE with a very tightly-bound calmodulin subunit. This different calcium/calmodulin stimulated Type 1 PDE has been separated and characterised by a number of investigators. (Strada *et al*, 1984).

Fraction	100μM EGTA	1mM calcium	1mM calcium + calmodulin	percentage stimulation
original homogenate	32 ± .5	46 ± 1	47 ± 3	150
Peak 1	2.2 ± .4	2.3 ± .1	2.4 ± .3	110
Peak 2	3.3 ± .2	7.5 ± .1	8.4 ± .3	250
Peak 3	8.5 ± 1.1	9.6 ± 1.2	11 ± .6	130
Peak 4	4.9 ± .4	5.5 ± .1	5.0 ± .6	110

Table 3.5. Calcium/calmodulin stimulation of isoenzymes.
Fractions from the column chromatography (16,24,30,42) and the original homogenate were assayed at 100μM cyclic AMP with 100μM EGTA, or 1mM $CaCl_2$, or 1mM $CaCl_2$ with 10 units of bovine brain calmodulin. Activities are quoted in units of pmoles/min/μl. The figures are given as the mean of three experiments (±S.E.M.).

No effect of 2μM cyclic GMP on 1μM cyclic AMP hydrolysis could be seen for any of the peaks. This could be due to the lack of this isoenzyme in the tissue or to the loss of the cyclic GMP sensitivity. Thompson *et al* (1979) reported that the inclusion of 30% ethylene glycol in the column separation buffers prevented the loss of cyclic GMP sensitivity that occurs for this isoenzyme on freezing. It is possible that the cyclic GMP-stimulated isoenzyme in olfactory

tissue is more labile than others. No success was, however, found when it was attempted to stimulate cyclic AMP hydrolysis with cyclic GMP in crude homogenates of sheep olfactory PDE. This latter observation, however, could have been due to the high levels of competing isoenzymes that would be present in a crude homogenate.

In order to further characterise the isoenzymes, the kinetic constants were obtained for the four peaks for both cyclic AMP and cyclic GMP over an appropriate ten-fold range of substrate concentrations. The determined kinetic constants are given in table 3.6. They suggest that the four isoenzymes all show roughly equivalent specificity for the two substrates.

Peak	cyclic AMP		cyclic GMP	
	K_m	V_{max}	K_m	V_{max}
1	3.8	0.32	0.83	0.67
2	2.4	5.0	1.3	4.0
3	110	7.0	22	7.4
4	4.0	1.4	7.0	0.8

Table 3.6. Kinetic parameters of separated isoenzymes.

Kinetic parameters were obtained by varying the substrate over an appropriate ten-fold concentration range. The units of V_{max} are given in pmoles/min/µl fraction and the units of K_m are given in µM. The assayed peaks were those pooled from the following fractions: 1 (14,15,16,17,18); 2 (23,24,25,26); 3 (29,30,31,32); 4 (40,41,42,43,44). Kinetic constants were estimated by the use of reciprocal plots and are the values obtained from single experiments. Where duplications were necessary, due to excessive scatter, good agreement between experiments was found.

The similarity of the kinetic constants for the first two peaks could be due to the first peak being formed by the action of proteolysis on the second which would have lost the calcium sensitivity (Tucker *et al*, 1981). Further work would be required to determine if this shoulder is a separate isoenzyme. PDE activity from rod cells exists as a single isoenzyme with K_d values for cyclic AMP and cyclic GMP hydrolysis of greater than 4mM and 150µM respectively. The lack of any

evidence of low affinity PDE's, or cyclic GMP-specific PDE's, in olfactory tissue suggests that such a PDE does not exist in olfactory tissue. The lack of a cyclic AMP-specific isoenzyme in olfactory tissue is quite surprising, as this isoenzyme often occurs in tissue preparations.

3.4. DISCUSSION.

3.4.1. Phosphodiesterase activity in olfactory epithelium.

Homogenates from both rat and sheep olfactory epithelium contain high levels of cyclic AMP and cyclic GMP PDE activity, relative to that of the brain. The lack of a suitable rat cilia preparation means that the activity of PDE in the actual olfactory cilia cannot be determined. The olfactory receptors are widely assumed to reside on the cilia or terminal swelling (Rhein and Cagan, 1981). One could argue that the PDE activity from the cilial fraction would be masked by that from the other olfactory tissue. It has been shown in the frog, for which a suitable cilia preparation is available, that the majority of adenylate cyclase activity of the total homogenate is present in the cilia preparation (Pace *et al*, 1985). The sonication procedure used for the preparation has been shown to yield a satisfactory odorant-stimulated adenylate cyclase, with a stimulated over basal ratio similar to that found by other investigators who used a cilia preparation (see chapter 2). It must be concluded that the sonication method used in chapter two and this chapter yields a preparation containing enzyme activities derived mainly from that of the cilia. It would seem likely that the majority of PDE activity in the rat sonicate would also derive from that in the cilia.

The failure of sonication to yield a satisfactory sheep PDE preparation meant that a total homogenate of the turbinate had to be used. It would be expected that a fairly large proportion of the total PDE activity in sheep would arise from non-cilial enzyme. The additional isoenzymes found in the sheep soluble fraction may be a consequence of this contamination. Pace *et al* (1985) found that the majority of adenylate cyclase activity of the total olfactory epithelium was found in the cilial fraction. Again, one would expect that this would also be the case for PDE activity and that the cilial PDE activity would represent a large fraction of the total activity. The lowered

specific activity of the sheep homogenate may have been due to the contamination of this preparation with soluble proteins from deep within the epithelium.

Specific antibodies against the various isoenzymes of PDE can easily be raised, given the ability to purify those isoenzymes to homogeneity (Hurwitz *et al*, 1984). These antibodies can then be used to probe for enzyme activity in the olfactory mucosa. Anholt *et al* (1987) used antibodies to show the differential location of the various G proteins across the olfactory epithelium. A similar study of the PDE isoenzymes would be very interesting. Kincaid *et al* (1987) used high affinity antibodies to Type 1 PDE to show that this calcium/calmodulin stimulated enzyme was found in specific neurones in the brain. This PDE isoenzyme was found predominantly in nerve cells, and so would be expected to be present in high concentration in the olfactory neurones. The authors, however, did not study cross sections of the olfactory bulb or olfactory epithelium.

A further study on the particulate PDE's in olfactory tissue is necessary to determine if the high activity found in olfactory homogenates is due to either a true membrane-bound enzyme, or to soluble enzyme trapped in membrane vesicles, and whether or not any particulate isoenzymes have any interesting properties that would have been masked in a total crude homogenate.

3.4.2. Relevance of olfactory phosphodiesterases to transduction mechanisms.

PDE isoenzymes found in olfactory tissue resemble those found in other tissues and do not seem to be unusual in their properties. The ion-exchange separation of soluble homogenate from rat olfactory homogenate shows the existence of two PDE isoenzymes one of which could be identified as a calcium/calmodulin-regulated Type 1 form. The ion-exchange separation of the sheep homogenate yielded three, possibly four, isoenzymes of which one, again, was a calcium/calmodulin-regulated Type 1 form. Differences between the elution profiles between sheep and rat were probably due to the different homogenisation methods employed. As described in the previous section the additional isoenzymes probably come from other cells in the epithelium such as sustentacular or basal cells. The non-Type 1 PDE isoenzymes in olfactory tissue need further study to determine if their lack of sensitivity to cyclic GMP stimulation is due

to proteolysis or whether they have no sensitivity *in vivo*.

The lack of any odorant-mediated effects specific to olfactory tissue at physiologically relevant concentrations of odorants seems to rule against the presence of a receptor-coupled PDE. The lack of any olfactory PDE isoenzymes showing similar kinetic constants and specificity to PDE from rod cells (Baehr *et al*, 1979), seems to suggest that a receptor-coupled PDE similar to that in rod cells does not exist in olfactory tissue. Anholt *et al* (1987) studied the location of various G proteins in olfactory epithelium by using specific antibodies. They found no evidence of the G protein of the rod cell, transducin, in olfactory epithelium, which again argues against the presence of this transduction mechanism.

The most likely task for the olfactory PDE, assuming the presence of an odorant-stimulated adenylate cyclase, would be in returning the levels of cyclic nucleotides back to resting levels. The presence of the calcium/calmodulin Type 1 isoenzyme in olfactory tissue allows a possible scheme to be devised. Binding of odorant to the receptor leads to stimulation of the adenylate cyclase. The increased levels of cyclic AMP then either activate cyclic AMP dependent protein kinases which then in turn activate ion channels, or directly activate the ion-channels. The membrane depolarisation that follows channel activation may lead to the release of calcium ions from intracellular stores which then activate Type 1 PDE and inhibit the adenylate cyclase enzyme (see chapter 2) in a potential feedback mechanism. These types of closed loop systems are discussed by Rasmussen & Goodman (1977).

The ability of calcium ions to stimulate PDE Type 1 activity could allow for possible interactions between cyclic nucleotide metabolism and other transduction mechanisms. The second messenger inositol trisphosphate causes the release of calcium from intracellular stores which could affect the activity of PDE (see section 1.4.1, in Chapter 1). Calcium ions could, therefore, be a possible link between receptor-coupled phosphoinositide metabolism and cyclic nucleotide concentrations. Interactions between the two systems could also result from the action of protein kinases stimulated by one system acting on the other system.

4. CHAPTER FOUR: THRESHOLD VALUES OF SOME LOWER FATTY ACIDS AND A DESCRIPTIVE SENSORY STUDY OF THEIR AROMA.

4.1. INTRODUCTION.

Psychophysics is defined as the branch of psychology describing, in quantitative terms, the relationship between physical stimuli and psychological response. As explained in the introduction, the lack of an *in vitro* test for olfactory function means that the structure of the olfactory receptors has had to be inferred from psychophysical studies of olfaction.

4.1.1. Threshold determinations.

One type of information that psychophysical studies can provide is an estimation of the thresholds of odorants. It should then be possible to deduce information about the receptors from the data obtained. The threshold of a compound will vary from measurement to measurement for the same subject, and from subject to subject. In order to circumvent these problems the threshold of an odorant is usually estimated by averaging the measurements from a large number of subjects. For practical purposes, the threshold is usually defined as that concentration which a person can detect in 50% of the trials on which it is presented. Two types of threshold measurements are recognised, namely the detection threshold and the recognition threshold. The detection threshold is the lowest concentration of an odorant that can be detected, whilst the recognition threshold is the lowest concentration of an odour that leads to correct identification. In practice the two threshold values will differ, since at low concentrations it is possible to detect an odour but not to identify it. The detection threshold is the easiest parameter to measure and is usually the figure that is quoted.

The response of a subject to a stimulus will be biased by the motivation and expectation of that subject. This observation led to the development of the signal detection theory (Green & Swets, 1966). This theory states that bias is inherent in experiments; the response of a subject

will depend on the experimental conditions as well as the concentration of the odorant. At low concentrations the subject will tend to produce a lot of false positive responses, dependent on the motivation of the subject, which have to be corrected to find the 'true' threshold. Techniques such as that used by Amoore (1970) avoid this problem by giving the subject a series of flasks. At least one flask contains the stimulus and the rest contain blanks. The probability of a subject guessing the location of the stimulus by chance is considerably reduced, over the situation that occurs when using an olfactometer in which the subject has a 50% chance of guessing the presence of a stimulus. Repeated trials are necessary in olfactometry in order to correct for 'false alarms' at low stimulus concentrations (Engen, 1982).

One of the many problems inherent in threshold determinations is the difficulty in estimating the concentration of the odorant in the inhaled air. Most investigators use sniff bottles to estimate thresholds, a technique described in great detail by Amoore (1970). In such a sealed vessel, the odorant will partition between the aqueous (or oil) phase and the air until an equilibrium value is attained. Normal breathing results in an inhalation of about 250 ml/sec of which only 5 to 10% flows past the olfactory cleft (Engen, 1982). This volume of air would deplete the headspace of even very large containers extremely rapidly. For this reason the concentration of odorant in the inhaled air will vary widely and most thresholds are reported in units of concentration in the solution. In a sealed vessel at equilibrium, the concentration of odorant in the air can be calculated from a knowledge of the air-liquid partition coefficient. However, this parameter does not stay constant with either concentration or temperature, hence this type of calculation can only give crude estimates of concentration. Air-water partition coefficients can be either measured directly, or calculated from data on solubility and saturated vapour pressure (Shirley, 1987).

Olfactometry allows the presentation of a constant concentration of the odorant in the air, but has two main disadvantages, firstly that complex and expensive instrumentation is needed, and secondly that threshold determinations are very time-consuming. A knowledge of the concentration of the odorant in the air is, however, still one step removed from knowing the all important concentration of the odorant in the mucus bathing the receptors; the air-mucus partition

coefficient will influence this concentration. When using a sniff bottle the concentration of odorant in the mucus will approach that of the aqueous solution (assuming that mucus behaves in a similar way to water) at equilibrium. In this case the air-water partition coefficient will not influence the threshold, but merely the time taken to reach equilibrium. Equilibrium is, however, impossible to achieve using sniff bottles, unless the sniff bottle is sufficiently large to allow the subject to be enclosed within it!

Various compilations of odour threshold values exist (Stahl, 1973; Fazzalari, 1978). It is found that for many common odorants, such as the fatty acids, numerous threshold values are published. The published values, however, vary widely over several orders of magnitude, due to the different techniques and dilution media employed by investigators. For a person trying to determine the variation of the threshold for a series of compounds, the odour compilations can be a source of much confusion. It is rare that any of the published threshold values can be compared with each other as most of the threshold studies that have been done tend to investigate only a few diverse odorants, and also use a variety of different methods which yield widely different answers. Compilations of threshold values will remain of dubious value until a set of standard techniques are used to determine them, which allow comparisons between studies.

Anosmia, or the inability to smell, can be due to several factors. The so-called specific anosmia (Amoore, 1977) which is anosmia for only a single class of odorants, is presumed to be due to a defect at the level of the receptor; one receptor class is either absent or functionally deficient. General anosmia can be due to either swollen turbinates preventing air flow past the olfactory epithelium, or an accident having sheared off the olfactory nerves at the cribriform plate. Hyperosmia, the heightened ability to smell, was reported by Henkin & Bartter (1966) who reported that people suffering from adrenal cortical failure (Addison's disease) were up to 100,000 times more sensitive to odorants than average. So great was this difference that the authors reported that no overlap of the thresholds was present between people with Addison's disease and the normal population. Treatment of Addison's disease sufferers with glucocorticoids resulted in their sensitivity returning to normal.

4.1.2. Magnitude estimation.

The threshold of a compound is a useful parameter, but in order to decide what significance a certain concentration of an odorant will have to a food or drink, a measure of how the perceived level of a compound changes with its concentration is also necessary. The technique of magnitude estimation, as it is called, has led to the discovery that the perceived intensity of an odorant with increasing odorant concentration can be described by the following equation (Stevens, 1975);

$$R = C\,(I - I_0)^n$$

where 'R' is the psychological intensity, 'C' is the arbitrary choice of units for odorant concentration, 'I' is the concentration of odorant, 'I_0' is the stimulus threshold and 'n' is an exponential term. The parameter 'n' is found to vary greatly in value from 0.07 to 0.7 depending on the odorant but is usually about 0.5 (Berglund *et al*, 1971). An 'n' value of less than one means that the rate of perceived intensity does not follow the concentration of the odorant; for example doubling the concentration will produce, usually, a perceived increase in intensity of only about one and a half times.

The perceived level of a constant concentration of an odorant will decrease with time, and this process is called adaptation. Much work has been done to decide if adaptation is a consequence of biochemical adaptation, or whether it is due to habituation (a process in which the brain progressively ignores the constant signal). The issue has not yet been resolved (Engen, 1982).

4.1.3. Odour Classification.

The simplest type of odour classification is one in which an odorant is grouped in with other similar odorants. The group is identified with a key word which is easily recognisable. The perception of colour is an ideal psychophysical model for studies of this sort. The three groups of stimuli in vision are the 'primaries' which consist of monochromatic light of three wavelengths, namely Red, Green and Blue. The three primaries in vision can be mixed in proportions to obtain any of the other perceived colours. Once analogous primary odorants have been identified, it

should be possible to search for the physico-chemical parameters which are analogous to wavelength. The identification of primary odour classes in olfaction has, however, met with little success. Harper *et al* (1968) reviewed attempts to generate classes of odorants; the number of classes ranges from a few up to a classification scheme devised by the authors that had forty-four classes. The number of classes generated will depend on the number and diversity of the odorants present in the study, and odour classification attempts of this type have generally been unsatisfactory. Classification systems such as that described by Harper *et al* (1968) are very useful as a starting point for the generation of descriptors for profiling. A typical classification system, in this case from Boelens (1974), is given in table 4.1.

Another approach to identifying odour classes is that proposed by Amoore (1977), in which he proposed a scheme to identify anosmic assessors by threshold measurements on the population. Some people have anosmia for only a certain class of compounds - these people are said to have a specific anosmia, to distinguish them from 'normal' anosmics who have a generalised defect in their ability to smell. Amoore (1977) presumed that this specific anosmia represents an inherited defect in one of the receptors. The difference between the threshold for the specific anosmics and that of the general population will be greatest for the odorant that shows the highest specificity for that receptor. This odorant can hence be considered as a primary odour. Various odour classes have been identified using this technique (Jennings-White, 1985). However, there is no evidence that the odour classes generated by this method have any more significance than those generated in other ways such as those described by Harper *et al* (1968).

The use of odour classes as the basis of descriptive analysis is widely used in the food and drinks industries (reviewed Powers, 1984). The technique involves the generation of a series of descriptors applicable to the product or products being assessed and the training of a panel in the consistent use of these descriptors. The products are rated by the assessors by scoring the level of each descriptor for each product, usually using a line scaling system, normally with a scale of zero up to a high level of the descriptor. The products, following descriptive analysis, can then be described in terms of the level of each descriptor present. Principal component analysis of the results (Smith, 1984) can form a map of the products, usually in two or three dimensions, in

Odour Class	Chemical Compound
Ethereal	Alkanes, alkenes, alkynes, alkyl halides, and nitro alkanes to about C5; alcohols to C3; ethers (linearly) to about C8.
Alliaceous	Straight chain thiols, thioethers, thials, thioketones, and thioesters; linear dialkyl-, dialkenyl-, di-, and trisulphides.
Green	Unsaturated linear alcohols, aldehydes, and esters from about C5 to C10; esters from about C2 to C10; linear ketones from C5 to C10.
Rancid (fatty)	Saturated linear aldehydes from C5 to C15; methyl ketones from C10 to C15; fatty acids from C4 to C15; alcohols and esters from C10 to C15.
Burnt (pyrotic)	Benzenoid hydrocarbons; phenols, cresols, xylenols, and lower ethers; substituted dioxofurans and pyrans.
Aromatic (spicy)	Substituted benzoid and alicyclic (medium ring) derivatives, with these substituents: hydroxyl, methoxyl, dioxymethylenyl, carbonyl, allyl, and propenyl.
Floral (fragrant)	Substituted (medium ring) cyclic and isosteric compound, with a carbon chain from about C2 to C8 and these functional groups: alcohol (esters), carbonyl, and carboxy (esters).
Woody	Two- or three-ring systems and isosteric compounds with about 12 to 17C atoms and these functional groups: alcohol (ester), carbonyl.
Musky	Macrocyclic (C14 to C18) and isosteric compounds with cross-sectional area of 50 Angstroms and a length-over-breadth ratio of 3.0; molecular weight of about 250.
Nauseous	Pyrrol, pyridine, chinoline, indole and lower homologues, steroids and isosteric compounds; organic diamino- and aminosulphide compounds.

Table 4.1 Relationship of chemical structure and perceived odour.
Taken from Boelens (1974).

which the distance between acids is a measure of the similarities between them. The axes of the maps can be easily labelled, as they usually correlate highly with one or more of the descriptors used. The main disadvantage of this method is the possibility of overlooking some important odour attribute, by not having a descriptor that represents it.

The difficulty of assessing odorants using classes has led some investigators to use the technique of Multi-Dimensional Scaling or MDS (MacFie & Thompson, 1984) coupled with similarity scaling. The use of MDS in olfaction has been reviewed by Schiffman *et al* (1977). During an MDS experiment the assessor is only required to rate pairs of odorants for similarity - no qualitative descriptors are necessary. An 'odour space' is then generated from the scores by the MDS analysis, in an analogous way to generating a road map from the distances between all the towns on the map. The method maps out the odorants in one or more dimensions. The closer odorants are to each other in space, the more alike their odours are. There are, however, several problems with this analysis. All odorants must be compared with all other odorants, pairwise and in both combinations, which generates a large number of samples for an assessor. Once a map has been generated, the axes of the map tend to resist labelling with any obvious parameter, though like/dislike quite often stands out (for an example see Schiffman (1974). The performance of assessors in discriminating between odorants depends on the differences between the odorants compared. An example of this is the study by Schiffman (1974) in which the fatty acids she studied did not differ greatly from each other. The differences between the fatty acids may have been obscured by the much larger differences between the other, diverse, odorants studied at the same time.

Despite the vastly different techniques of descriptive analysis and MDS, very similar results are usually obtained. Williams & Arnold (1985) compared the two methods, along with that of free-choice profiling, on a series of coffees, and obtained very similar results. The use of free-choice profiling is a relatively new technique in which the assessor is allowed to generate and use his or her own descriptors. Procrustes analysis (Gower, 1975) of the results 'rotates' each assessors odour space so that all assessors are mapped in a similar way. Procrustes analysis has the advantage that the lengthy training of descriptive analysis and the lengthy experimental procedures of similarity scaling are not necessary. The analysis also has the advantage that individual assessor differences are not ignored, and so will usually be more sensitive than the other two techniques. Williams & Arnold (1985) also used Procrustes analysis on the descriptive analysis results and obtained improved results, presumably as this allowed the expression of

assessor individuality. The main disadvantage of this method is that the necessary computer software is not readily available.

The confirmation of whether the odour classes identified by psychophysical methods represent true primary odorants or not, will probably have to wait until the receptors can be purified by biochemical techniques and the binding of a series of odorants investigated.

4.1.4. Investigations of fatty acids.

The fatty acids ranging from formic acid (C1) to decanoic acid (C10) are an interesting series of odorants. The odour of the acids changes dramatically from a stinging and vinegary smell for the first three acids, to a smell resembling sweaty socks for the middle acids and to a meaty and fatty complex smell for the higher members of the series. The 'sweaty' smell is unique to the carboxylic acid group - no other odorant classes can duplicate this distinctive odour. As odorants, the fatty acids are somewhat unusual in having an ionisable group (the carboxylic acid function) and hence relatively high water solubility. This results in high water/air partition coefficients (Shirley *et al*, 1987b) and thus low volatility, which probably explains the relatively high thresholds of these compounds. Many threshold values are published in odour threshold compilations (Stahl, 1973; Fazzalari, 1978). These values are widely variable due to the many different methods and dilution media employed by investigators. Meaningful studies are those in which the thresholds for a whole series of odorants are determined, since the change of threshold along a homologous series can then be investigated. Such studies on fatty acids have been carried out by Laffort (1969) and Amoore (1970).

The unique 'sweaty' smell coupled with the charged carboxylic acid group suggests the possibility of a unique receptor for the fatty acids. This hypothesis is strengthened by Amoore's discovery of specific anosmia for this odour type (Amoore 1970). The carboxylic acid group creates complexities. In order to gain access to the olfactory mucosa the acid must escape the liquid phase and to do so must be in the protonated form. Once the acid reaches the olfactory mucosa it will dissolve in the mucus and, as the mucus will probably have a pH in excess of its pK_a, will exist predominantly in the ionised form. The pH of the mucus and of the stock solution

will, therefore, influence the threshold. The acids have an advantage over many other odorants in that they are almost infinitely stable.

There are numerous examples of fatty acid odorants in industry; the importance of acetic acid as a flavour in the confectionery business is obvious to anyone who eats salt and vinegar flavoured crisps. Fatty acids are important in the flavour of whisky and it has been shown that acetic, butyric, iso-valeric, hexanoic and octanoic acid are of importance (Jounela-Eriksson, 1977). The fatty acids are quite often associated with 'off' flavours of food and a study by Vorbeck *et al* (1961) showed that concentrations of fatty acids from propionic to hexanoic were elevated in the cheesy smelling 'off' sauerkraut, as compared with the fresh food. Fatty acids are also of importance in the flavour of bread (Johnson *et al*, 1966) with acids from formic to octanoic being found, and in the flavour of many drinks (Konigsbacher & Donworth, 1966). Probably the most well known occurrence of the fatty acids is in dairy products, with the sweaty smelling acids being important in the flavour of butter and cheeses (Day, 1966). A review by Pryde (1979) lists many of the sources of some natural fatty acids; an interesting example is the presence of relatively large amounts of octanoic acid in coconut (Downing, 1961).

Whether or not human pheromones exist has long been debated (Gower, 1988). The supposed vaginal pheromone (copulin) of the rhesus monkey, which is produced at oestrus, is capable of mediating profound behavioural effects in these monkeys (Michael *et al*, 1971). The major component of copulin was found by the authors to be a mixture of medium chain length fatty acids. The same authors also found copulin-like extracts from women near the middle of the menstrual cycle providing a hint that copulin or something similar may be a pheromone for humans (Michael *et al*, 1974). Some evidence supporting this view came from a study by Cowley *et al* (1977) which showed that copulin and some steroids had some effects on the perception of subjects during social interaction. The changing olfactory sensitivity of women during the menstrual cycle (Doty *et al*, 1981), and the observation that menstrual cycles tend to match up in women room-mates (McClintock, 1971) also tends to support the hypothesis that some kind of pheromone is involved in human behaviour. Much further work is required in order to demonstrate that human pheromones exist and whether or not fatty acids, in particular, play a

role.

In view of the importance of the fatty acids as odorants in our lives it is surprising that more psychophysical studies of their odour have not been done. Amoore (1970) studied the thresholds of fatty acids from formic to decanoic acid and some branched chain acids. He found that the thresholds of the acids dropped to a minimum for butyric and valeric acid, with the lowest threshold obtained for iso-valeric acid. Amoore carried out his studies in buffered solutions at the pK_a of the acid. This is important, as the higher the proportion of the protonated form, the lower the threshold, due to the greater concentration of the volatile species. For this reason, thresholds of acids must be measured in buffered solutions at a constant pH, which is usually chosen to be the pK_a of the acids. At a pK_a value of about pH 3.8, both protonated and unprotonated forms will be present in equal concentrations. Laffort (1969) also studied the thresholds of the straight chain fatty acids, but presents his data as air concentrations, hence direct comparisons with the results of Amoore (1970) are not possible.

Many studies on diverse odorants have been carried out, in which a fatty acid is included in the study. I was, however, unable to find any psychophysical studies on the change of the odour of the fatty acids with chain length. Amoore *et al* (1972) have studied some lower fatty acids by using direct similarity scaling as part of their study of the specific anosmia to iso-valeric acid. Amoore only compared the odour of his acids to that of iso-valeric acid and not with each other, therefore an MDS analysis could not be performed. This type of scaling gives an idea of the similarity of the acids to each other, but gives no indication of the odour qualities of the acids. Schiffman (1974) studied many of the fatty acids used in this report as part of a study of a large series of odorants, using the method of similarity scaling and coupling it to Multi-Dimensional Scaling (MDS) in order to summarise the results. The map which was thus obtained showed little difference between the fatty acids, presumably because the differences between the fatty acids themselves were slight, compared to the differences between the fatty acids and other odorants.

4.1.5. Aims.

The lack of information on the odour of the fatty acid odorants and their importance to my

industrial sponsors, Unilever PLC, led to the decision to investigate their properties using the psychophysical techniques in use at Colworth house, Unilever. It was decided to use a sophisticated spinning-band-still, at Warwick, to purify the acids to a high degree. In olfaction, highly pure odorants are necessary as a small contamination with a highly volatile or 'potent' odorant could invalidate the results. The fatty acids are more prone to contamination than other odorants as they tend to have higher thresholds than many other odorants. They do, however, have the advantage that once purified they will remain stable. When the purification of the acids was completed, the project was moved to Colworth house. Fatty acids in the range formic to octanoic acid were used, since the higher acids are not so important as flavours. Additionally, the 'standard' sweaty smelling fatty acid (iso-valeric acid), was also included. Two or three cyclic acids were also included, as these rather unusual fatty acids had not previously been studied.

Descriptive analysis is widely used at Colworth for assessing food products. It was decided to use this method for the assessment of the fatty acids; results from this analysis would be more applicable to industry. It was envisaged that the results from the descriptive analysis could be summarised using varying statistical techniques and any correlations between the results and physico-chemical parameters searched for.

The thresholds of the acids used in the study were determined using the method of Amoore (1970) to determine the 'reproducibility' of threshold determinations. Thresholds measured as concentration in an aqueous phase are more applicable to industry than thresholds reported as air concentrations, since it is necessary to know what concentration of odorant in a product will be detected by a consumer. The results from the threshold determinations could also be investigated for any physico-chemical correlations.

4.2. MATERIALS & METHODS.

4.2.1. Chemicals.

The eleven fatty acid odorants used in this study were obtained from Aldrich (Gillingham,

Dorset, U.K.) and were of the highest commercially available purity. Formic, acetic and octanoic acid were used as supplied but the other acids were further purified as described in the next section.

Analytical grade potassium hydrogen phthalate was purchased from Aldrich. 100ml Quickfit conical flasks and stoppers were obtained from Gallenkamp (Loughborough, Leicestershire, U.K.). Double-distilled odour-free water was used to make up all solutions.

4.2.2. Estimation of purity of fatty acids.

The purity of the acids was estimated by using a 0.25 inch, 9 foot FFAP (free fatty acid phase) column from Phase Separations Ltd (Queensferry, Clwyd, U.K.) installed in a Pye-Unicam 104 gas chromatograph. Temperatures of up to 180°C were needed to elute the higher fatty acids within 20 minutes. Acids were injected into the column neat as 0.5µl aliquots using a Hamilton 7001 micro-syringe. Percentage impurities were obtained by measuring areas of the contaminating peaks and comparing them to the sample peak. Correction for relative flame ionisation detector (f.i.d.) response was not done as it was not feasible to identify the impurities. Formic acid does not produce an f.i.d. response so its purity could not be determined - no significant impurity peaks could be identified and the compound was not further purified. Acetic acid also showed no significant impurities (Aldrich quote the level of formic acid impurity to less than 0.01%) and was not further purified. Octanoic acid was estimated to have a purity of about 99.94% as supplied and was not further purified. All the other acids had significant levels of impurity and were further purified. Estimated purities before distillation were as follows: propionic acid (99.8%), butyric acid (99.5%), valeric acid (99.7%), hexanoic acid (=98%), heptanoic acid (=97%), iso-valeric acid (99.5%), cyclo-butane carboxylic acid (99%), cyclo-pentane carboxylic acid (98%) and cyclo-hexane carboxylic acid (99%).

The major contaminant in iso-valeric acid is the 2-methyl isomer which has the same listed boiling point (Aldrich admit the contamination at an unknown level). The FFAP column used could not resolve the two isomers so a 0.3mm, 13 foot OV1 capillary column fitted to a Carlo-

Erba 'Fracto-Vap' gas chromatograph was used. Various column and injector temperatures were tried but only a partial resolution of a 50/50 mix of the two acids could be obtained. The resolution achieved was not sufficient to show up any 2-methyl butyric acid contamination in the iso-valeric acid sample. A longer column may have been successful but was not available. The difficulty of separating the two isomers probably explains the lack of a quoted impurity of the 2-methyl isomer from Aldrich. Due to these problems it is not known whether or not the spinning band still managed to separate out the two isomers. The 2-methyl isomer smells quite different to iso-valeric acid but has a considerably reduced intensity, hence a small impurity of this isomer would probably not be a problem in the following experiments.

4.2.3. Purification of fatty acids.

The acids were purified by distillation using a Perkin-Elmer model 251 Auto Annular still. This still has the advantage that compounds which differ in boiling point by only a couple of degrees can be separated. This separation can be achieved due to the rapidly rotating and very close fitting teflon spiral inside the machine, which results in about two hundred theoretical plates. The high efficiency is a consequence of the extremely large area for liquid to vapour equilibration that the tight fitting, rapidly rotating band produces.

Propionic acid was distilled at atmospheric pressure, all other acids were distilled under vacuum due to the shortening of band life that occurs when temperatures greater than 150 °C are used. Precise regulation of vacuum is required to maintain the efficiency of the still. A Quickfit 'Bibby' vacuum regulator was used rather than the very expensive Perkin-Elmer item, as a vacuum harder than a few mm of mercury was not required. An adjustable bleed was introduced between the vacuum regulator and the still to prevent jerky regulation of pressure, when a vacuum softer than about 10 mm of mercury was needed. The bleed also served to correct for leakage past the valve of the vacuum regulator, which tended to produce a gradual increase in pressure when a large differential between still pressure and vacuum pump pressure existed.

Approximately 30-50 ml of the neat acid was required per distillation in order to produce about 10-20 ml of purified acid. The majority of the impurities were of lower boiling point than

the acids themselves, so the distillation consisted of setting a slow reflux (approximately 10 drops per minute) and taking off the acid enriched in the contaminants at a rate of about 1 drop every 5 minutes, until the contamination dropped below the desired level. When the distillate was sufficiently pure, the take-off rate was increased to drive off the purified acid. The fractions were monitored constantly by gas chromatography. When the distillation was finished the purest fractions were pooled and stored at -20 °C until needed.

Several problems were noticed when using the spinning-band machine: the most serious problem stemmed from the use of teflon for the majority of components. Teflon is well known for being inert, but what is not well known is its ability to absorb compounds. The previous user had distilled a compound containing a sulphide residue and had followed the instructions with regard to cleaning, that is to distil a large volume of acetone through the still. The acetone did not remove all of the compound, which subsequently led to contamination of distillate. Washing the still with other solvents was also unsuccessful. The only solution was to dis-assemble the machine after every distillation, remove the worst of the contamination by washing in a suitable solvent, and treat all components in a vacuum oven at 100 °C over a weekend under vacuum pump pressure. The remainder of the machine was then filled up with a mild detergent for a few hours and rinsed with acetone. No problems resulted from the gentle curving required to get the teflon spinning band into the vacuum oven. These precautions would not normally be necessary, but the nose can be more sensitive than a gas chromatograph and compounds not showing on the gas chromatograms can still be detected. Smelling the components after cleaning served as a useful check for cleanliness. The lengthy cleaning process coupled with the distillation itself taking two days meant that only one distillation could be done in a week.

It was found that the fatty acids tended to 'creep' past the reflux valve; quite large volumes could be taken off with the reflux valve turned off. It is important to correct for this leakage since too high a take-off could upset the equilibrium in the still head. Another problem was that most of the acids tended to mist against the 'window' which meant that the reflux rate was difficult to determine. It is not known whether this problem is unique for the fatty acids; it is not mentioned in the manual. A replacement direct drive plug was manufactured with two O-rings rather than

the original one which held the plug more firmly in the glass-ware. In addition a finer thread was cut in the retaining plug which tended not to loosen as much as the original design.

The distillations using the Perkin-Elmer still were usually very successful with an improvement in purity of up to 100 times from a single distillation. Figure 4.1 shows a typical distillation, with heptanoic acid illustrated. The distillation of cyclo-pentane carboxylic acid was not successful. The fractions coming off the column smelled very different to the starting material, having an additional ester/sweet smell which did not correlate with the partial disappearance of any of the impurity peaks, which were impossible to remove to any large extent. The most likely explanation was that the acid was not stable during the distillation. However, the acid should have been stable at the 120 °C used for distillation. Contamination of the still was also a possibility except that the additional smell present was not that of a recognisable fatty acid. It was decided to remove cyclo-pentane carboxylic acid from the studies because of the doubts as to what was contributing to its odour. All other acids that were distilled showed no obvious difference in odour between the starting material and the purified fractions. The following purities of the other acids were obtained: propionic acid (99.97%), butyric acid (99.98%), valeric acid (99.99%), hexanoic acid (99.85%), heptanoic acid (99.93%), iso-valeric acid (99.98%), cyclo-butane carboxylic acid (99.97%) and cyclo-hexane carboxylic acid (99.94%).

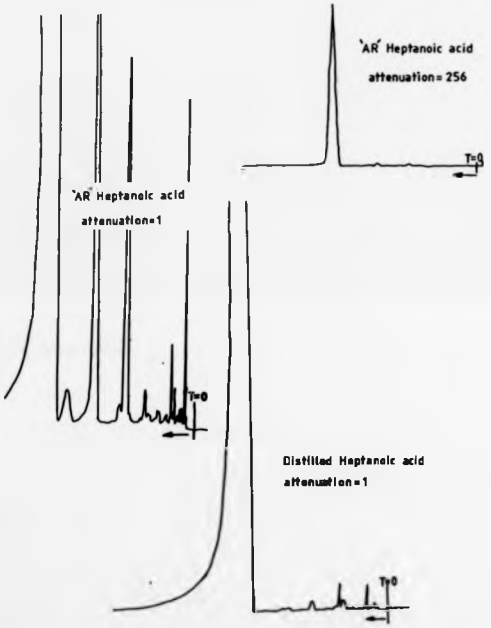
4.2.4. Determination of Threshold of Detection.

Subjects for threshold determinations were volunteers from the staff at Unilever, Colworth. Subjects were male or female and aged between eighteen and forty years.

The threshold determination used a 'two out of five' forced choice procedure as used by Amoore (1970). This technique involves the subject being presented with seven sets of five flasks. Two of each set of flasks contain the odorant and the other three contain an odour blank. The seven sets of flasks contain a range of concentrations expected to encompass the probable threshold of the subject. The subject is required to pick two flasks out of each set which he thinks have a different odour from the rest (the chance of the subject guessing correctly is only 1/10). The subjects sniffed from low to high concentrations of the acid in order to minimise adaptation.

Figure 4.1. Gas chromatography traces showing the improvement obtained by spinning band distillation of heptanoic acid.

Gold label heptanoic acid from Aldrich was distilled using a Perkin-Elmer spinning band still as described in the text. Purity of the acid was assessed using an FFAP gas chromatography column at 180 °C as described in the text. The results show the f.i.d. response of 0.5µl aliquots of the original acid at two attenuation levels and the response of an aliquot of the pooled fractions, subsequent to distillation, at the lower attenuation.



No standard was given to the subjects beforehand, so they were not familiar with the odour they had to detect.

Acids were made up in 0.1M potassium hydrogen phthalate buffer, with the pH adjusted to the pK_a of the particular acid. Conical flasks of 100 ml were used, with two of each set of five flasks containing 10 ml of diluted acid in phthalate buffer, and the other three containing 10 ml of phthalate buffer as an odour blank. Quaternary dilutions were used in order to encompass the expected threshold range of the population in seven sets of flasks. Amoore has found that the threshold of a population for a particular odorant tends to vary over a range of 256 fold and in his experiments he used binary dilutions (Amoore, 1970). The problem with binary dilutions is that more than one set of flasks is required to encompass the expected range of a population. A short ranging experiment would be required for each assessor in order to estimate which set of flasks would encompass his or her threshold. Due to pressure of time it was decided to use quaternary dilutions for the determination of threshold in this study as the threshold for the great majority of the population would be expected to fall within a range of seven dilutions. All concentrations were expressed as ppm total acid.

Each subject was given seven sets of flasks, with each set being in a different booth. The threshold measurements took place under white light in air conditioned booths (19 °C to 22 °C). The subject was required to indicate which two of the five flasks in any set had a different odour from the others. It was not possible to instruct the subject to indicate which flasks had a detectable odour, due to the presence of a slight background odour of the glass flasks that could not be eliminated by washing. The subjects were not allowed to go back and smell earlier sets of flasks, but they were allowed to sniff in whichever manner suited them. Each estimation took about ten minutes. Between twenty and twenty-five subjects participated in each determination. This number is lower than most studies but sufficed to give a good estimate of the mean of the population. A major problem with studies of this type is obtaining sufficient volunteers for an experiment. People are usually willing to give up time for a 'one-off' experiment but are less willing to give up time on a regular basis. The threshold for each person was taken to be the lowest concentration at which subsequent correct answers were given. An example of a threshold

form with a typical response is given in figure 4.2.

After an experiment the flask contents were neutralised using a sodium hydroxide solution, rinsed with running water and soaked over a weekend in a 5% Decon-90 detergent solution. Following this, the flasks were rinsed firstly in running water for a few hours, then in double distilled water. The flasks were finally soaked in double distilled water for a couple of hours then allowed to drain and dry upside down on absorbent paper. The flasks were checked individually for any excessive residual odour and those flasks smelling differently from the majority were rejected.

4.2.5. Sensory evaluation.

It was decided to use the technique of quantitative descriptive analysis (QDA) for the series of fatty acids as reviewed by Powers (1984). The technique basically consists of generating a series of descriptors to describe the compounds under test, the descriptors should be able to distinguish between all products and cover all variation. Assessors are then trained to use the descriptor list in a consistent way. The training consists of the use of test compounds, to obtain panel agreement, these usually contain defined levels of the standard for each descriptor. Obviously, odour studies of compounds rather than food products are much harder as the compounds themselves are usually used as the standards. Training aims to remove as much variance due to the scoring procedure as possible. The actual scoring is done by using a line scale, preferably unstructured but usually marked out in categories.

4.2.5.1. Orientation and profile generation.

For the initial periods twenty-four assessors, who were volunteers from the staff at Unilever, Colworth, participated. Most of the assessors had prior experience of profiling techniques for food products. Several sessions, taking the form of round table discussions, preceded the main experiment. Thirteen assessors with a proven reliability record were selected for the final experiment.

Descriptive terms were developed as follows;

Figure 4.2. Threshold form used during the threshold measurements; as completed by a volunteer.

Volunteers were instructed to circle two numbers corresponding to the two flasks in each set that they thought had a different aroma from the others. The concentration of acid in the flasks rises in concentration by four times with each set of flasks going from the first to the seventh set. The forms are marked after the experiment and the threshold for that volunteer taken as the concentration from which all subsequent scores were correct.

CODE:- 13 NORM:- ✓ STRONG:- WEAK:-

THRESHOLD TEST.

SECTION (1). PLEASE FILL IN OR RING AS APPROPRIATE.

DATE:- 19.07.86 TIME:- 10.00 a.m.

NAME:- Rachel Colebold AGE:- 18

SECTION:- 741

~~MALE~~/FEMALE DO YOU SMOKE? YES/~~NO~~

HAVE YOU HAD A COLD IN THE LAST WEEK? ~~YES~~/NO

ARE YOU SUFFERING FROM A NASAL ALLERGY AT THE MOMENT? YES/~~NO~~

SECTION(2).

BOOTH	FLASKS	
1	A (B) X C D (E) X	763 ppm
2	A (B) X C (D) X E	305 ppm
3	A (B) ✓ C (D) ✓ E	122 ppm
4	A B (C) ✓ D (E) ✓	48[illegible]
5	(A) ✓ (C) ✓ D E	1950 ppm (3)
6	(A) ✓ (C) ✓ E	7810 ppm
7	A (B) ✓ D (E) ✓	31,200 ppm

[1] All assessors were presented with samples of the acids in 100ml conical flasks and were prompted to describe the aroma of those acids. The descriptions were initially generated on an individual basis followed by a group discussion. A slightly different procedure of asking the assessors to note differences between a set of three different acids was also used; this produced some additional comments.

[2] The descriptors were combined and presented at subsequent sessions along with suitable references to the descriptors, where assessors could identify them. Assessors were prompted to indicate which descriptors applied to which acids. Rarely used, or confusing descriptors were deleted or combined at this stage, leaving a manageable list of about thirty descriptors.

[3] The thirteen assessors used for the final experiment were then presented with the list of descriptors and the corresponding references. Further discussion led to elimination and combination of some descriptors resulting in a final list of twenty five which are presented in figure 4.3 along with their references.

Due to the lack of time, further training of the assessors was not possible. In an experiment of this type, one would normally aim to conduct training experiments to determine if the subjects score the attributes in a similar way to each other, and then to concentrate on those descriptors for which variation is present or those assessors that score the descriptors in a different way to the majority (Powers, 1984). It was found that it was impossible to get a consensus for all the assessors as to whether or not a particular descriptor was present for each acid. It was hoped that the consequent panel variation would not obscure the variation due to the different acids and that sufficient descriptors were included to allow the full differences between the acids to be visualised. The lack of a reproducible rating for many of the descriptors is clearly reflected in the results.

4.2.5.2. Aroma assessment.

The eleven acids were diluted in the phthalate buffer used for the threshold determinations, with the exception of formic, acetic and propionic acid. These acids were diluted in distilled

Figure 4.3. List of the descriptors, and their definitions, as obtained during the training of the assessors for the descriptive analysis.

FATTY ACID DESCRIPTOR DEFINITIONS.

1. INITIAL IMPACT	The time taken from sniffing to perception of odour.
2. VINEGAR	The odour of a 10% vinegar solution .
3. CARAMEL/CARAMAC	The odour of the Caramac candy bar.
4. MOTHBALLS	The odour of mothballs.
5. STINGING/SHARP	The non-odour sensation associated with mineral acids such as hydrochloric acid.
6. STILTON	The odour of stilton cheese.
7. MATURE CHEDDAR	The odour of mature cheddar cheese.
8. SWEATY SOCKS	The odour of mature socks that have been worn by sweaty feet.
9. AMMONIA	The odour of 1% household ammonia or ammonia solution.
10. NAPPIES	The odour of wet nappies soaking in Nappisan detergent.
11. WET CARDBOARD	The odour of wet and old cardboard.
12. MUSTY/OLD BOOKS	The musty odour of old books.
13. MOULDY	The odour of bread mould.
14. VINYL	The odour of new vinyl associated with a paddling pool.
15. PEARDROPS	The odour of peardrops sweets.
16. BOILED SWEETS	The odour of hard boiled fruit sweets.
17. APPLE	The odour of freshly sliced apple.
18. COCONUT	The odour of fresh coconut.
19. FATTY/CANDLES	The fatty odour of candles.
20. FATTY/TWEEN	The fatty odour of Tween detergent.
21. SOAPY/TEEPOL	The soapy odour of Teepol detergent.
22. FLASH LIQUID	The odour of Flash pine cleaning liquid.
23. TOILET BLOCKS	The odour of Scentinal toilet blocks.
24. OVERALL INTENSITY	The overall odour impression.
25. PERSISTANCE	The time that the odour "lingers" after sniffing.
26. ADDITIONAL	Please add any other odour descriptor that you can detect and score it accordingly on the scale.

water, due to the very high concentrations that had to be used in the experiment. All the acids were adjusted to approximately equal odour intensities using 500ppm iso-valeric acid as the standard. This was accomplished using the assessors' comments during the training periods. The concentrations used are included in the caption to table 4.3. The same acid solutions were used throughout the experiment to eliminate any variation due to acid concentration.

Samples were presented to the assessors in the same booths used for the threshold determinations. A partially balanced incomplete block experimental design with randomised coding was generated using an 'in-house' program. The balanced design is needed to ensure equal replication of each acid and to ensure that the replications of each acid are spread out over the sessions and assessors. The partially balanced design resulted from the replacement of one assessor mid-way through the experiment due to illness. The incomplete block design is necessary as there are more samples than the assessors can smell in one sitting; it was decided that four was the highest number of samples that would be given at one time. The experimental design called for a series of six sessions which were spread out over two weeks. The design needed twelve samples, so hexanoic acid was replicated. Each of the twelve assessors had four different samples per session in a randomly coded order with a different labelling code for each session to ensure that the assessors could not deduce the identity of the acid. Each acid was replicated twenty-four times with twelve assessors per session. An example of the first side of a coding form for a single acid is presented in figure 4.4. The sheets were coded for the session, acid, assessor number and order by use of adhesive labels generated by the experimental design program. These labels were stuck at the top right hand side of the sheet. The intensity of each descriptor was scored by the assessors on a standard ten point interval scale with '1' representing a zero intensity and '10' representing a high level. Completed forms were then entered into a database by two typists and errors corrected by looking for differences between the two sets using another 'in-house' program. The codes that identified the acid for each session were then decoded and replaced by a variable identifying the acid. It was found that, despite instructions to the contrary, some assessors did not label the '1' on the sheet but left it blank when none of the attribute was present. It was decided on the basis of this observation to code all missing values as

'1'.

4.2.6. Statistical analysis.

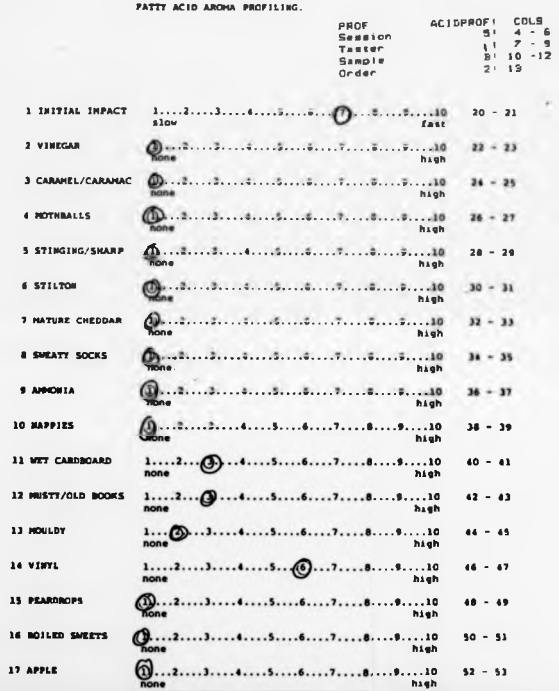
The sample size from the threshold determinations was too low to check meaningfully for a Gaussian distribution, so simple means and standard errors of the means (SEM's) were calculated. In order to look for possible structure activity relationships a database of various simple physical and chemical parameters was collected from the 1987 Aldrich catalogue and the Handbook of Chemistry and Physics (45th edition). The SAS procedure RSQUARE (SAS, 1985) was then used to generate suitable regression equations using the physico-chemical parameters and the threshold scores. The database and SAS commands used are given in the appendix.

The panel scores obtained from the profiling experiment were assumed to be independent observations from normally distributed random variables. A two-way analysis of variance (ANOVA) was performed using 'sample' and 'assessor' as the independent variables in order to compare means and interactions. The SAS procedure GLM was used to perform the calculations (SAS, 1985). The commands used and the database are presented in the appendix. Duncan's test was then performed on the significant descriptors to see which means differed significantly from each other. As the observations were acquired during six independent sessions a three way ANOVA using 'session' as an independent variable should have been performed. It was found in preliminary analyses that no significant differences were obtained between acids for the different sessions and that only slight differences were obtained between the assessors response for the different sessions. It was, therefore, decided not to use the session number as a variable in the analyses, in order to simplify the results.

For comparison purposes an ANOVA was performed using adjustment of the assessor means by the 'lsmean' option of the GLM procedure. This operation was carried out to try to reduce some of the variance between assessors. The mean scores of those assessors who consistently scored higher were reduced whilst those assessors scoring lower than average had their mean scores increased. The two sets of data were compared.

Figure 4.4. The first side of the coding form used, for the descriptive analysis of the acids, by the assessors.

The form was on two pages; the second page contained the remainder of the descriptors. Each assessor had four sets of sheets for each session corresponding to the four acids to be assessed. The sheets were coded for the assessors by the stickers that were stuck in the top right hand side of the form. The stickers allowed identification of the session, assessor, acid and the order that the acids were presented to the assessor. The assessor ringed that number on the scale that he/she felt quantified the level of that descriptor in the sample.



The differences between the acids were visualised using principal component analysis (PCA) of the mean scores using the SAS procedure FACTOR (SAS, 1985). The SAS commands used are given in the appendix. In order to reduce the number of variables input to the analysis, which requires that there are fewer variables than samples, descriptors with a significance value P of greater than 0.001 were discarded. In addition, some descriptors correlated with an 'r' value of greater than 0.90, and so the 'best' of them were selected by choosing the descriptor with the highest range of values, or that descriptor with the highest 'F' value. Some descriptors were also rejected as they had lower 'F' values than others. This left 10 descriptors to be used in the PCA. The varimax rotation option was used in the analysis to simplify the labelling of the axes. This was accomplished by rotating the axes in space until the highest correlations between the axes and individual descriptors was obtained. The SAS procedure RSQUARE was used to try to generate regression equations between the first three PCA dimension scores for the acids and the relevant physico-chemical parameters. The SAS commands used and the database are presented in the appendix.

For further information on analysis of variance and principal component analysis see Hinkle *et al* (1979), Green (1978) and the SAS statistics manual (version 5: 1985).

4.3. RESULTS.

4.3.1. Thresholds of the fatty acids.

The thresholds for the fatty acids (presented in table 4.2) drop to a minimum at around butyric acid, then rise again. The threshold value for the branched chain iso-valeric acid is lowest, with cyclo-hexane carboxylic acid only slightly higher. The standard errors of the mean are similar to those reported by Amoore (1970). The distribution of the assessor thresholds are given in figure 4.5. The distribution of the acids seems to be approximating to Gaussian in each case. There is no sign of any specific anosmics for iso-valeric acid though the sample sizes used here would be unlikely to detect the low incidence of 1% (Amoore, 1970). The distribution of thresholds for cyclo-hexane carboxylic acid shows some sign of having a bi-modal distribution

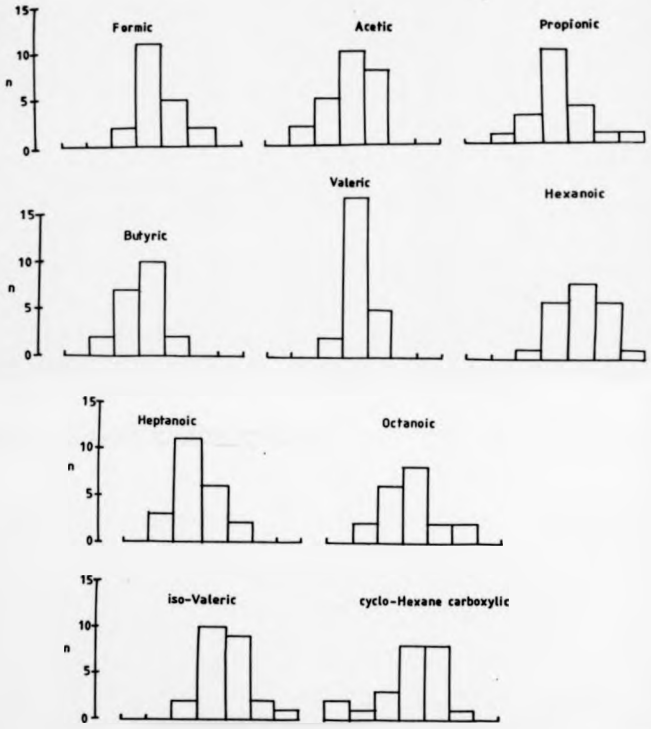
though this is not significant with the sample size present.

Acid	n	log(threshold) ± SEM	Threshold ppm
Formic	20	3.8 ± 0.13	6000
Acetic	25	2.6 ± 0.12	460
Propionic	20	2.8 ± 0.16	640
Butyric	21	0.58 ± 0.11	3.8
Valeric	24	0.92 ± 0.06	8.4
Hexanoic	22	1.6 ± 0.13	43
Heptanoic	22	1.7 ± 0.13	46
Octanoic	20	1.1 ± 0.16	14
iso-Valeric	24	0.041 ± 0.12	1.1
cyclo-Hexane carboxylic	23	0.26 ± 0.17	1.8

Table 4.2. Thresholds of the fatty acids.
The thresholds were obtained using a 'two out of five' forced choice procedure. Acids were buffered at pK$_a$ using 0.1M phthalate buffer and were presented as quaternary dilutions. The logarithm of the thresholds are quoted as ± standard error of the mean. Thresholds are presented as ppm total acid. The number of subjects per determination is given as the quantity 'n'.

The threshold values show a similar trend to those of Amoore (1970), but they are approximately one order of magnitude higher than reported by him. The thresholds obtained in this report are compared with those of Amoore (1970) in figure 4.6. The values reported by Laffort (1969) also agree in terms of group trends with the values reported here, and those of Amoore. The results of Laffort (1969) cannot be directly compared to those reported here as aqueous concentrations, since his thresholds are quoted as concentration in the air. Conversion of Laffort's results to units of ppm in the aqueous phase using the air-water partition coefficient is not possible as that assumes equilibrium in the system used by Amoore and in this report - this would not be a valid assumption. There is no published value for a threshold for cyclo-hexane

Figure 4.5. The distribution of the thresholds of the various acids.
The level of each bar represents the number of subjects that had a threshold corresponding to that set of flasks. The sets go from low to high concentration in quaternary steps.



carboxylic acid, hence no comparisons can be made with this acid. The two sets of data in figure 4.6 differ with respect to octanoic acid. Amoore (1970) reported a rise in threshold from heptanoic to octanoic acid followed by a subsequent drop in threshold for the higher acids, whilst I found a drop in threshold from heptanoic to octanoic acid. These differences may be attributed to slight differences in techniques and to the relative imprecision of these types of measurements. Laffort (1969), despite using a different technique, found similar trends of threshold with chain length and found a large drop in threshold from heptanoic to octanoic acid.

It is apparent from the comparison of these results with those of Amoore and Laffort that it is the group trends, and not the absolute values which are important. Reported thresholds in the literature tend to vary over many orders of magnitude (Stahl, 1973; Fazzalari, 1978). They will be dependent on the technique used and should be used only to get a rough idea of a threshold value. Useful studies would include a series of odorants, with perhaps a 'standard' such as iso-valeric acid used by all investigators. This would allow easy comparisons between groups. The difference between the threshold value for iso-valeric acid quoted in this report and that given by Amoore (1970) is broadly mirrored for the other acids in the series.

I attempted to correlate the thresholds of the acids with physical and chemical parameters by using regression analysis. For this purpose, the SAS procedure 'RSQUARE' was used. This program selected the best model for each number of variables from 1 to 'n', where 'n' is the number of physico-chemical parameters. The SAS procedure used, along with the database, is presented in the appendix. It was found, when applying the analysis to the straight chain acids, that using 4 parameters gave a good fit of the data (R^2=0.982) and that using 5 parameters gave an almost perfect fit (R^2=0.995). The two models are presented here;

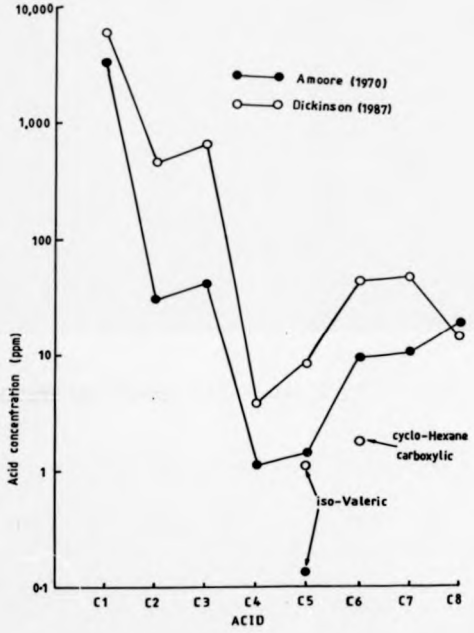
[VP (vapour pressure, defined as the temperature which gives a pressure of 1mm Hg); MP (melting point); Dens (density); Diam (diamagnetic resonance)]

1. $log\,(threshold) = -109.8 + 9.81Pk_a + 0.0562VP - 0.0223MP + 64.04Dens$

2. $log\,(threshold) = -111.7 + 10.39Pk_a - 0.0462Diam + 0.08493VP - 0.02MP + 64.97Dens$

Applying the models to the relevant physico-chemical parameters for iso-valeric acid gave

Figure 4.6. Comparison of the results obtained in this study with those obtained by Amoore (1970).



predicted thresholds of 0.15ppm and 0.07ppm respectively, which are much lower than the experimental values. The predicted values for nonanoic acid are 140ppm and 99ppm respectively and for decanoic acid 350ppm and 230ppm respectively. The calculated values for nonanoic acid and for decanoic acid do not fit in the threshold values for these acids as shown by Amoore (1970); he found a lowering of threshold of these two acids as compared to octanoic acid. Therefore, it must be concluded that whilst the two models generated fit the experimental points, they are not useful for predicting the thresholds of other fatty acids. The SAS program 'RSQUARE' generated a very large number of other models for the regression. The models shown above are the best ones for four and five variables. (It is quite possible that some of the other models with four, five or even six variables would estimate thresholds for iso-valeric, nonanoic or decanoic acid with more success than the above models). The large number of successful models generated by the analysis suggests that some simple relationship between threshold and physico-chemical parameters is not present.

4.3.2. Profiling results of the fatty acids.

The results of the analysis of variance (ANOVA) of the results with assessor and acid as independent variables and the descriptors as the dependent variables are shown in table 4.3. A significant result for the main effect 'assessor' shows that some assessor means are significantly different from others. A significant result for the main effect 'acid' shows that some acid means are significantly different from others. The analysis does not indicate which assessors differ from each other, or which acids differ from each other for each descriptor. The interaction term in table 4.3 is defined as whether the level of one of the independent variables (assessor) affects the dependent variables (descriptors) in the same way across the levels of the second independent variable (acid). A significant result shows that the same interaction is taking place between 'acid' and 'descriptor'. A significant interaction term shows that all the assessors do not use that descriptor in a consistent way. A common cause of interaction is the assessors not scaling intensities in the same way, usually due to assessors using different parts of the scoring scale (Powers, 1984). Confusion on the part of the assessors as the the definition of the descriptor

would also lead to interaction. The hope with an experiment of this sort is that the variability in the assessor scores do not obscure the variability due to the acids.

Further training or Procrustes rotation (Gower 1975) might have reduced some of the panel variability. Williams and Arnold (1985) used Procrustes rotation on profiling results and obtained an improvement. No Procrustes software was available so the technique could not be used. The lsmean option of the 'GLM' program in SAS allowed some correction for variability of assessor response by reducing the mean of assessors consistently scoring higher than the others. The two sets of data differed only slightly so the corrected means were not used for any further analyses. The high assessor variation meant that for many of the poorer descriptors, the variance due to the assessor was much greater than that due to the acid, which would have obscured some information. The descriptors used for the PCA analysis, however, had F values for the acids similar to, or greater than the F values of the assessors.

Figure 4.7 is a Duncan's test on the significant descriptors from table 4.2. The immediate problem that arises from this sort of analysis is the difficulty in interpretation. No descriptor is capable of distinguishing between all the acids, as one might expect, and some descriptors are more successful than others. An ideal descriptor would be one in which the majority of the scoring scale had been used and a large range of scores obtained. The low ranges of many of the descriptors coupled with high assessor variation led to fewer significant differences.

In order to summarise the data more clearly it is necessary to know which descriptors are highly correlated and hence give some redundant information. Figure 4.8 shows the correlations between the descriptors used in this study. Some of the descriptors were highly correlated, for example vinegar (no. 2) and sharp (no. 5). The fruity descriptors peardrops (no. 15), boiled sweets (no. 16) and apple (no. 17) were also highly correlated. The cheesy/sweaty descriptors also showed high correlations. Knowing the correlated descriptors narrows down the number of descriptors that have to be studied to allow a summary of the data.

The statistical method called principal component analysis (PCA) is very useful for this sort of complex data. The variables fed into the analysis are recombined to form new ones called

Figure 4.7. Duncan's multiple range test (5% significance level) on the significant descriptors from table 4.3.

The output was produced using the 'GLM' procedure of SAS. Acids sharing a line are not significantly different from each other at the 5% significance level. Mean intensity scores, for the acids, for each descriptor are shown also. The acids are coded as follows:

(1) formic acid; (2) acetic acid; (3) propionic acid; (4) butyric acid; (5) valeric acid; (6) hexanoic acid; (7) heptanoic acid; (8) octanoic acid; (9) iso-valeric acid; (10) cyclo-butanecarboxylic acid; (11) cyclo-hexanecarboxylic acid.

Descriptor 2:- Vinegar

7.04	7.00	6.42	3.33	3.17	2.96	2.83	2.62	2.13	2.13	2.00
3	2	1	9	4	5	7	10	8	11	6

Descriptor 3:- Caramel

2.54	1.83	1.75	1.58	1.58	1.50	1.50	1.25	1.17	1.13	1.13
11	9	7	10	6	5	3	1	8	2	4

Descriptor 4:- Mothballs

3.29	2.71	2.00	2.00	1.92	1.50	1.37	1.37	1.33	1.33	1.29
6	8	11	7	5	9	4	2	10	3	1

Descriptor 5:- Sharp

7.21	6.29	6.25	3.54	3.46	3.33	3.25	3.23	2.91	2.83	2.75
1	2	3	9	5	4	7	6	11	8	10

Descriptor 6:- Stilton

3.67	3.54	3.00	2.54	2.29	1.96	1.71	1.33	1.25	1.08	1.08
9	5	4	6	11	8	10	7	3	1	2

Descriptor 7:- Cheddar

4.87	4.13	3.88	2.67	2.63	1.90	1.79	1.54	1.21	1.17	1.08
9	4	5	11	10	6	8	3	7	1	2

Descriptor 8:- Sweaty socks

4.63	4.17	3.00	2.92	2.88	2.63	2.50	2.42	1.92	1.25	1.04
9	5	4	10	11	7	6	8	3	2	1

Descriptor 10:- Nappies

3.04	2.42	2.42	2.29	2.21	2.21	2.08	1.83	1.58	1.50	1.50
9	6	5	11	7	4	8	3	2	10	1

Descriptor 11:- Wet cardboard

2.21	1.88	1.79	1.63	1.33	1.29	1.21	1.21	1.08	1.04	1.00
7	11	8	6	5	9	3	10	4	2	1

Descriptor 12:- Musty

4.13	3.42	3.08	2.17	2.08	1.83	1.63	1.58	1.33	1.21	1.17
8	6	7	5	11	4	9	3	2	10	1

Descriptor 13:- **Mouldy**

2.96	2.88	2.79	2.63	2.58	2.46	2.38	2.00	1.63	1.38	1.29
5	6	11	7	8	9	4	10	3	2	1

Descriptor 14:- **Vinyl**

4.50	4.10	3.96	3.29	2.29	2.13	1.96	1.92	1.42	1.38	1.33
7	6	11	8	5	4	9	10	3	2	1

Descriptor 15:- **Peardrops**

3.54	2.63	1.67	1.54	1.54	1.50	1.39	1.25	1.08	1.08	1.00
11	10	9	7	4	3	6	5	8	2	1

Descriptor 16:- **Boiled sweets**

4.33	4.25	1.96	1.63	1.54	1.46	1.46	1.38	1.29	1.19	1.08
11	10	9	4	5	7	3	8	1	6	2

Descriptor 17:- **Apple**

3.29	3.08	2.08	2.08	2.04	1.88	1.71	1.63	1.50	1.29	1.15
11	10	4	3	2	9	5	7	8	1	6

Descriptor 18:- **Coconut**

1.88	1.67	1.50	1.38	1.27	1.21	1.17	1.08	1.08	1.04	1.00
11	7	9	3	6	8	5	4	10	1	2

Descriptor 19:- Fatty (candles)

2.79	2.29	2.27	2.13	1.29	1.21	1.13	1.08	1.08	1.00	1.00
7	8	6	11	9	4	1	3	2	10	5

Descriptor 20:- Fatty (Tween)

2.04	1.58	1.58	1.50	1.46	1.38	1.21	1.21	1.17	1.13	1.00
7	8	11	6	9	4	2	5	3	1	10

Descriptor 21:- Soapy (teepol)

2.75	1.71	1.67	1.67	1.54	1.44	1.29	1.21	1.13	1.08	1.04
8	9	4	7	11	6	5	3	10	2	1

Descriptor 23:- Toilet blocks

2.35	2.17	2.13	1.83	1.50	1.46	1.33	1.29	1.29	1.21	1.08
6	5	11	8	7	3	2	4	9	10	1

Descriptor 24:- Overall intensity

7.63	7.25	6.50	6.50	6.46	6.44	6.33	6.29	6.21	5.67	5.54
1	3	2	4	9	6	10	5	11	8	7

Descriptor 25:- Persistence

6.00	5.58	5.00	5.00	4.92	4.79	4.67	4.63	4.40	4.37	4.04
3	1	2	4	9	11	10	5	6	8	7

Figure 4.8. Correlation matrix of the descriptors used for the profiling of the fatty acids.

The correlation matrix was generated on the mean acid scores by the 'FACTOR' procedure of SAS. The correlation coefficients are coded as follows with negative coefficients in parentheses:

* 0.90≤ r ≤1.00; + 0.80≤ r <0.90; - r <0.80

Descriptors are coded as follows:

(1) Initial impact; (2) Vinegar; (3) Caramel; (4) Mothballs; (5) Sharp; (6) Stilton; (7) Mature cheddar; (8) Sweaty socks; (9) Ammonia; (10) Nappies; (11) Wet cardboard; (12) Musty; (13) Mouldy; (14) Vinyl; (15) Peardrops; (16) Boiled sweets; (17) Apple; (18) Coconut; (19) Fatty (candles); (20) Fatty (tween); (21) Soapy (teepol); (22) Flash liquid; (23) Toilet blocks; (24) Overall intensity; (25) Persistance.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	*	-	-	*	-	-	-	-	-	-	-	(+)	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
4	-	-	-	*	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-
5	-	*	-	-	*	-	-	-	-	-	-	-	(+)	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	*	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	+	*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	+	+	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	*	-	-	+	-	-	-	-	*	+	-	-	-	-	-
12	-	-	-	+	-	-	-	-	-	-	-	*	-	-	-	-	-	-	+	-	-	-	-	-	-
13	-	(+)	-	-	(+)	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	+	-	-	*	-	-	-	-	*	+	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	+	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	*	*	-	-	-	-	-	-	-	-
18	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-	*	+	-	*	-	-	-	-	*	+	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	*	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-
23	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-
24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	+
25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	*

Descriptor	Main effect				Interaction	
	Acid		Assessor		Acid*Assessor	
	F	Sig	F	Sig	F	Sig
1) Initial impact	0.46	ns	8.50	***	1.15	ns
2) Vinegar	32.40	***	9.58	***	0.99	ns
3) Caramel	5.12	***	28.18	***	1.21	ns
4) Mothballs	8.90	***	14.04	***	1.40	*
5) Sharp	26.27	***	21.49	***	2.01	***
6) Stilton	21.20	***	26.09	***	6.54	***
7) Mature cheddar	21.51	***	7.58	***	2.84	***
8) Sweaty socks	17.59	***	20.64	***	3.14	***
9) Ammonia	1.96	ns	17.14	***	1.84	***
10) Nappies	3.50	***	26.74	***	1.35	*
11) Wet cardboard	4.59	***	14.50	***	1.42	*
12) Musty	16.82	***	17.51	***	1.90	***
13) Mouldy	6.35	***	23.41	***	1.82	***
14) Vinyl	22.61	***	20.04	***	2.74	***
15) Peardrops	14.35	***	11.01	***	2.44	***
16) Boiled sweets	29.12	***	13.08	***	3.15	***
17) Apple	13.45	***	23.94	***	3.48	***
18) Coconut	3.80	***	25.25	***	1.39	*
19) Fatty/candles	9.06	***	11.95	***	1.52	***
20) Fatty/tween	3.26	***	8.89	***	1.30	ns
21) Soapy/teepol	8.02	***	11.03	***	2.87	***
22) Flash liquid	0.93	ns	5.59	***	0.89	ns
23) Toilet blocks	3.50	***	7.91	***	1.02	ns
24) Overall intensity	3.65	***	9.39	***	2.42	***
25) Persistence	3.04	**	24.77	***	1.47	*

Table 4.3. Two way analysis of variance of the odour profile means of the fatty acids.

*Type 1 significance test results are shown. Significance level (Sig): *** $p<0.001$; ** $p<0.01$; * $p<0.05$; ns = not significant ($p>0.05$). Parameters were calculated using the 'GLM' procedure of SAS. The F values are given also. The dependent variable 'Acid' has 10 degrees of freedom, the dependent variable 'Assessor' has 12 degrees of freedom and the interaction term 'Acid*Assessor' has 100 degrees of freedom. The concentration of the acids (in ppm) used in the study are as follows: Formic (140,000), acetic (50,000), propionic (8,000), butyric (3,000), valeric (2,500), hexanoic (625), heptanoic (1,000), octanoic (1,000), iso-valeric (500), cyclo-butane carboxylic (5,000), cyclo-hexane carboxylic (5,000).*

principal components which are not correlated with each other. The reduced number of variables can be studied much more easily. The analysis basically makes linear combinations of the original descriptors, that are highly correlated, and forms the first principal component and then repeats the process with other principal components. The analysis is very useful for summarising a lot of data in which much redundant information is present. For meaningful results from the

analysis it is necessary to have more samples than variables. It was necessary, therefore, to choose ten descriptors from the 23 in figure 4.7. As described in the methods section this basically consisted of rejecting some descriptors highly correlated with others and rejecting descriptors with low significance or low ranges. Ten descriptors were obtained in this way, and were used in the PCA analysis.

PCA analysis of the ten 'best' descriptors produces a series of values of each descriptor for each principal component. The use of a SAS scoring procedure on these results then gives values for each acid. The best way of summarising this data is to plot out the dimensions. The PCA analysis gives the most significant principal component first followed by the next and so on until ten principal components are obtained which account for all of the original variance, in this case. The first two or three principal components usually account for the vast majority of the variance with other principal components only contributing a small amount to the results. PCA analysis of the above results indicated that the first three principal components accounted for 94% of the variance. Plotting out the first three principal components allows a summary of the data in which only a small amount of information has been lost; a disadvantage dramatically outweighed by the simplification of the results. The first three principal components are presented in the form of a three dimensional plot in figure 4.9.

When presented in this form, the profiling data is much easier to interpret than the results from Duncan's test; it is readily apparent that the acids split into four groups on the basis of the descriptors used.

The first three acids cluster tightly together with propionic acid tending towards a somewhat more complex odour than the other two. The 'sweaty' smelling fatty acids (4,5 & 9) cluster together with iso-valeric and valeric acid being very close together. Butyric acid lies slightly away from the other two. The two cyclic acids (10 & 11) differ quite a lot from the others with respect to the third dimension and differ from each other with respect to the first and second dimensions. The remaining three acids (6,7 & 8), with the more complex and difficult to describe odour characteristics, form the final group with heptanoic acid slightly displaced from the other

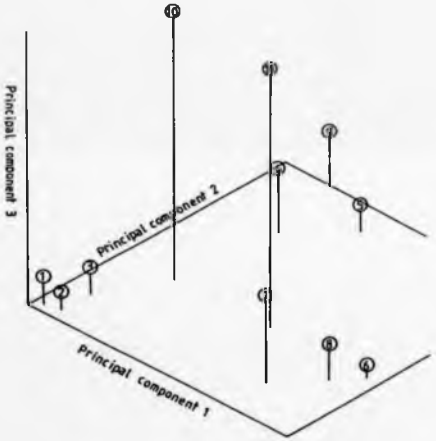
Figure 4.9. Three dimensional map of the mean acid scores, for the first three principal components, as obtained from the PCA of selected descriptors.

The following ten descriptors were selected for principal component analysis according to the text:

(2) Vinegar; (4) Mothballs; (5) Sharp; (6) Stilton; (7) Mature cheddar; (8) Sweaty socks; (12) Musty; (13) Mouldy; (14) Vinyl; (16) Boiled sweets.

The mean scores of the above descriptors for each acid were input to the 'FACTOR' procedure of SAS. The first three principal components, which accounted for 94% of the variance, were plotted as the scores for each of the acids, which were coded as follows:

(1) formic acid; (2) acetic acid; (3) propionic acid; (4) butyric acid; (5) valeric acid; (6) hexanoic acid; (7) heptanoic acid; (8) octanoic acid; (9) iso-valeric acid; (10) cyclo-butane carboxylic acid; (11) cyclo-hexane carboxylic acid.



two.

The varimax rotation option employed allows the dimensions of the PCA map to be labelled easily. The first dimension correlates highly with the more complex odour descriptors necessary to describe octanoic, hexanoic and heptanoic acid. Descriptors highly correlated with this dimension include mothballs, musty, mouldy and vinyl. The second dimension seems to represent the level of the 'sweaty' note and the third dimension to the 'fruity' note as typified by the cyclic acids. The descriptors vinegar/sharp correlated negatively with both the first and second dimensions.

The results shown indicate very little difference between the odours of hexanoic and octanoic acid, and indeed looking at the Duncan's test output shows that the two acids usually have very similar mean scores. In fact, the two acids do have noticeably different odours, to me, when compared with each other. The lack of difference on the PCA map is a consequence of using descriptors that do not distinguish between the two acids in the analysis. The only descriptor that was useful for this acid was number 19 (Fatty/candles) in which octanoic acid was significantly different from all the other acids. The descriptor, when input to a PCA analysis, produced a fourth dimension that represented the level of this attribute. The failure of other descriptors to distinguish between hexanoic acid and octanoic acid might be due to confusion by the assessors about the descriptors. This might be resolved by further training. Alternatively, the two acids may have had similar levels of the descriptors used. A search for other descriptors that may distinguish between hexanoic acid and octanoic acid could possibly have resolved the problem.

The lack of difference between the three 'sweaty' smelling fatty acids is not surprising. Comparing the acids together shows a great similarity between the odours with butyric acid smelling slightly different from the other two. Some of the differences between the 'sweaty' acids on the PCA map may be a consequence of slightly different intensities of the standard solutions. Descriptor 24 (overall intensity), however, shows no significant differences in the assessors perceived intensity of the 'sweaty' acids.

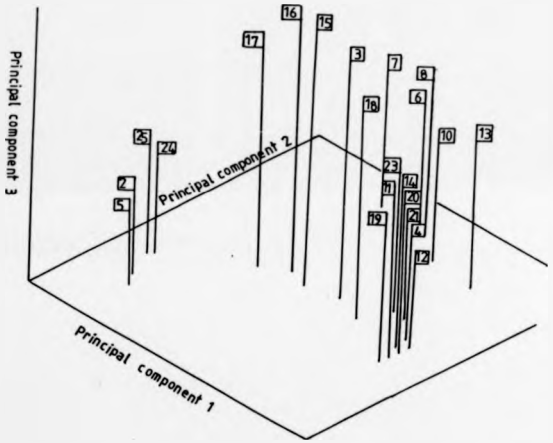
One might have expected from comparisons of the odour of acetic acid with formic and propionic acid that much greater differences would be apparent. This could be attributed to lack of suitable descriptors or insufficient training. Formic acid has a very simple odour and is considered to be a 'classic' trigeminal odour (Silver & Maruniak, 1981). This group of three 'stinging' acids will be those that have a high contribution from the trigeminal system. The descriptors stinging/sharp and vinegar are the only obvious descriptors for these acids. Some assessors observed that formic acid has a 'caramel' note to it but this was not reflected in the results. The lack of difference seen on the PCA map between the first three acids is probably, therefore, due to insufficient training and confusion on the part of the assessors as to the precise meaning of the descriptor. A possibility is that two sub-sets of assessors are present, each rating the odour of, say, acetic and formic acid in an opposite way. The results would, therefore, cancel each other out and when the assessor responses were averaged would show no differences between the two acids. Comparison of the means of the descriptors vinegar and stinging/sharp for each assessor, however, showed that all assessors tended to rate the first three acids with similar scores. The conclusion is that further training would be needed, probably involving side to side comparison of the 'vinegary' acids, in order to distinguish between the three acids more on the PCA map. Propionic acid has a hint of a 'sweaty' note to it and was rated higher than the other two acids for those the 'sweaty' and the other more complex descriptors. This resulted in propionic acid being shifted more towards the middle of the PCA map.

It is possible to view the relationships between the descriptors by analysing all 25 descriptors using principal component analysis and plotting out the levels of each principal component for each descriptor. The results, whilst not being statistically 'correct', gave a good indication of the relationship between the descriptors. The first three dimensions from this analysis are shown in figure 4.10. It must be borne in mind that the first three dimensions of this analysis account for only 75% of the total variance so a certain amount of information has been lost. The position of the descriptors on the map broadly correlate with the position of the acids in figure 4.9 and it can readily be seen that the vinegar and sharp descriptors lie in a similar position to that of the first three acids.

Figure 4.10. Three dimensional map of the factor loadings, for all of the descriptors, following principal component analysis.

The mean scores of the acids for all of the descriptors were input to the 'FACTOR' procedure of SAS. The factor loadings of the descriptors for each of the first three principal components, which accounted for 75% of the variance, were plotted out. The descriptors were coded as follows:

(1) initial impact; (2) vinegar; (3) caramel; (4) mothballs; (5) sharp; (6) stilton; (7) mature cheddar; (8) sweaty socks; (9) ammonia; (10) nappies; (11) wet cardboard; (12) musty; (13) mouldy; (14) vinyl; (15) peardrops; (16) boiled sweets; (17) apple; (18) coconut; (19) fatty/candles; (20) fatty/tween; (21) soapy/teepol; (22) Flash liquid; (23) toilet blocks; (24) overall intensity; (25) persistence.

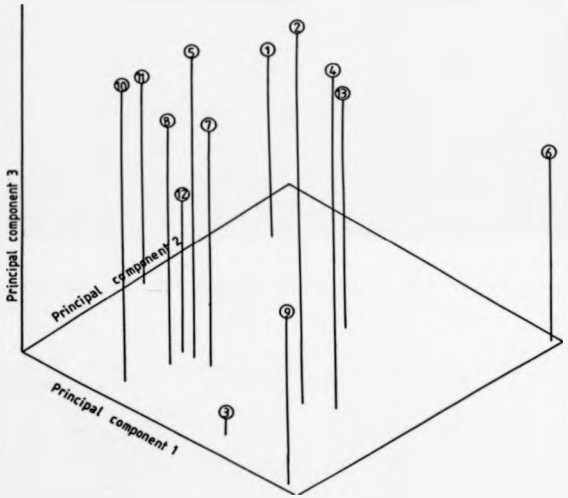


The variation of assessor response can also be summarised by the use of principal component analysis. The mean scores for each assessor can be calculated and input to the PCA analysis in a similar way to the mean acid scores. The first three dimensions from this analysis are represented in figure 4.11. These dimensions, however, account for only 72% of the variance, therefore some information has been lost. The assessors tend to cluster in one broad group except for assessors '3', '6' and '9' who are somewhat separate from the main group. Looking at the scores for the assessors it is obvious that assessor '6' tended to use much more of the scoring scale than the others and often rated a descriptor with the maximum value of ten. Further training would be necessary to bring his scores in line with the other assessors. Assessor '9' also tended to score many of the descriptors higher than the majority of the panel. Assessor '3' tended to disagree with the rest of the assessors for some of descriptors; soapy/teepol, vinyl and stinging/sharp were underscored compared with the overall assessor means whilst the fruity descriptors were in contrast given much higher scores than the other assessors (except for assessor '6'). These differences in scoring probably explains the displacement in position of assessor '3' on the PCA map. It may have been possible to bring assessor '3' into agreement with the rest of the panel as that assessor may have had different views on what some descriptors represented.

I attempted to demonstrate structure-activity relationships in a similar way to that tried for the threshold measurements. Correlation coefficients showed that the first principal component correlated (correlation coefficients > 0.90) with those physico-chemical parameters that increased smoothly with chain length, such as molecular weight and boiling point. The second and third principal components did not correlate highly with any of the physico-chemical parameters. Regression analysis on the first principal component, not surprisingly, showed that high correlations (R^2> 0.90) could be obtained while using just one independent variable; the independent variables used were those rising smoothly with chain length. Only small increases in the R^2 value were obtained with models using a larger number of independent variables. Regression analysis on the second and third principal components showed that at least five independent variables were required in order to obtain an R^2 value of greater than 0.90. The use

Figure 4.11. Three dimensional map, of the mean assessor scores, for the first three principal components obtained from the PCA of selected descriptors.

The mean scores for the same ten descriptors that were used for Figure 4.9, averaged over assessor, were input to the 'FACTOR' procedure of SAS. The first three principal components, which accounted for 72% of the variance, were plotted as the scores for each of the thirteen assessors.



of six independent variables, however, gave R^2 values very close to 1.0.

As with the regression analysis of the threshold data very many models can be generated. The first principal component, however, seems quite strikingly to represent the molecular weight of the compound, with formic acid having the lowest value and octanoic acid having the highest value. The sample size of only eleven acids is too small for the many regression models containing five or six independent variables to have any significance. It again seems likely that the probable interaction of the fatty acids with multiple receptors would invalidate any attempts to demonstrate structure-activity relationships using these methods.

4.4. DISCUSSION.

4.4.1. Threshold results.

The results show that despite the variability of thresholds reported in the literature, it is possible to replicate other results with a fair degree of success. Despite the low number of subjects that participated in the measurements, good comparisons with the results of Amoore (1970) were obtained. Knowledge of the threshold of an odorant in an aqueous solution will be useful in determining if the level of that odorant, detected by gas chromatography, is likely to be significant as part of its perceived flavour. Thresholds in aqueous solution cannot, however, be considered to be the same as would be found in other media such as food and drinks. Partitioning of the acid into different phases of a food will alter the threshold. The pH of the food or drink will also alter the threshold of the acid; a pH much higher than the pK_a of the acid will result in a dramatic increase in threshold as the proportion of the volatile protonated form becomes less. Due to the likelihood, however, of threshold trends within a group remaining constant for a variety of different dilution media it should only be necessary to measure a threshold for the new system for one of the acids and then infer the thresholds of the remaining acids using data from studies such as the one reported here.

The majority of evidence (see chapter 1) seems to suggest that multiple olfactory receptors are present with broad specificity. The fatty acids show dramatic changes in odour when

increasing in chain length. It is almost certain that the odour change is due to the interactions of the acids with several receptors. 'Classic' structure-activity work in pharmacology works with the interaction of a series of ligands with the same receptor (Martin, 1978). It would not be expected that a simple relationship between threshold and physico-chemical parameters would exist when the series of acids interact with more than one receptor. The ability to produce a model with only four physico-chemical parameters that fits the data well is not surprising given that the shape of the threshold curve is basically an inverted 'bell' shape. Most physical and chemical parameters change gradually upwards or downwards in magnitude with chain length and the combination of only two of these would produce this 'bell' shape. The very large number of successful models supports this view.

Laffort (1969) deduced a regression term that predicted the thresholds of a series of fatty acids, alcohols, esters and hydrocarbons with an r^2 correlation coefficient of 0.9, indicating a fairly good fit. His regressor term contained contributions from the molar volume, a hydrogen bonding index, atomic volume polarisability and the air-water partition coefficient. His final regression equation gave predicted values of threshold within one or two logarithmic units.

The observation that the shape of the threshold curve for the straight chain fatty acids goes though a minimum at around valeric acid was taken by Amoore (1970) to be evidence that there is a receptor fairly specific for this size of fatty acid. Amoore found a low incidence of anosmia for the 'sweaty' fatty acids. This anosmia was greatest for iso-valeric acid and Amoore suggested that iso-valeric acid was a primary odour. Shirley *et al* (1987b,c) found biochemical evidence that was consistent with this hypothesis. They found that the lectin Concanavalin A (Con A) blocked, fairly specifically, the EOG response in the rat for a single receptor that seemed to be fairly specific to straight chain odorants of four to six carbon atoms. Their results of the relative blocking of the EOG signal with chain length is shown in figure 4.12. The general shape of the graph, coupled with the position of iso-valeric acid and cyclo-hexane carboxylic acid, resembles that found for the human thresholds (figure 4.6). Shirley *et al* (1987b,c) found that the receptor seemed to be of importance for the detection of thiols and hydrocarbons of the above size also. It is possible to conclude that the major receptor involved in the detection of the lower fatty acids is

the so-called 'sweaty' receptor. The survival of the EOG of propionic acid and heptanoic acid was found to be similar in figure 4.12. The observation that the threshold for heptanoic acid is, in fact, much lower than propionic acid, suggests that another receptor is becoming important for the detection of these higher fatty acids, which was not observed in the experiments of Shirley *et al* (1987b,c). It is encouraging that biochemistry performed using laboratory animals can, for at least this example, be compared with psychophysical experiments with human volunteers.

4.4.2. Profiling results.

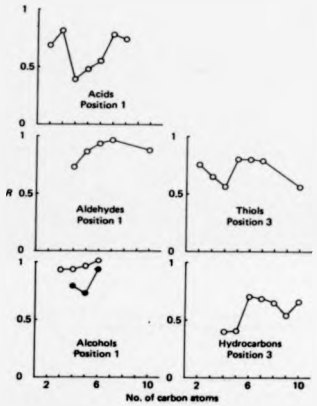
The results of the descriptive analysis of the fatty acids are quite good considering the small amount of training of the assessors. The variation due to the assessor response did not obscure the differences between the acids except for only a few of the descriptors and there were sufficient 'good' descriptors to allow a meaningful analysis of the results. Principal component analysis allowed the similarities between the acids to be viewed in the form of a three dimensional map. The map showed the presence of four broad groups of the acids which may indicate the involvement of four receptor classes. The results allow a summary of the main odour parameters of the fatty acids studied, presented in table 4.4.

It has been shown by Williams and Arnold (1985) that conventional profile analysis as used here produces similar results to both similarity scaling and to free-choice profiling followed by Procrustes rotation. The advantage of conventional descriptive analysis is that the dimensions of the PCA maps can easily be assigned to the descriptors, the main disadvantage being the more time-consuming training periods. Direct similarity scaling has the additional advantage that it is not possible to 'miss' an attribute. In addition confusion by assessors about a descriptor may lead to that descriptor 'missing' differences between the acids. Similarity scaling has the disadvantage that a very large number of sample assessments are needed in order to compare all acids pairwise in both combinations, and for more than a few samples the technique becomes too time-consuming.

The advantage that Procrustes rotation may have had is apparent when assessors score differently from each other. Assessors '3', '6' and '9' in the profiling scored some of the

Figure 4.12. Variation of R with chain length for the various homologous series of straight-chain saturated aliphatic compounds.

R is the fraction of an odorant's EOG response which survives Concanavalin A treatment divided by the fraction if the isopentyl acetate EOG response which survives Concanavalin A treatment. In the 'Alcohols' panel: open circles represent the 1-ol isomers and the filled circles represent the 2-ol isomers. Taken from Shirley et al (1987b).



Acid	Odour description
Formic	Vinegar, stinging/sharp.
Acetic	Vinegar, stinging/sharp.
Propionic	Vinegar, stinging/sharp.
Butyric	Sweaty.
Valeric	Sweaty.
Hexanoic	Mothballs, musty, vinyl, fatty.
Heptanoic	Wet cardboard, musty, vinyl, fatty.
Octanoic	Mothballs, musty, fatty, soapy.
iso-Valeric	Sweaty.
cyclo-Butane carboxylic	Sweaty, fruity.
cyclo-Hexane carboxylic	Sweaty, fruity.

Table 4.4 Estimated odour descriptions of the fatty acids studied in this report.
The descriptions are deduced from figure 4.7. The descriptions do not give any indication of the levels of each which are present. Only the major contributions to the odour are given.

descriptors in a different way, either by using the scoring scale in a different way, or by disagreeing as to the presence or not of a particular attribute. If an assessor is consistent within him/herself then free-choice profiling coupled with Procrustes rotation will result in similar ratings to the other assessors. Averaging the scores, as was done in this report, will tend to lose some information.

Attempts to demonstrate any structure-activity relationships between the perceived odour and physico-chemical parameters was not very successful. The small sample size of eleven acids hindered the study but the interaction of the acids with multiple receptors was probably the main reason the study was not successful. One striking result was the high correlation of principal component 1 in figure 4.9 with those physico-chemical parameters that increased smoothly with chain length. It is possible that at least some of the perceived 'signal' could be due in part to the

interpretation by the brain of a simple chromatographic model influencing odorant access to various parts of the epithelium - though the results from the threshold determinations would seem to suggest that such a simple system does not exist.

The observation that the acids cluster into four groups could be taken as evidence for the existence of four receptor classes. However, it is not known at what point the receptor classes are formed as there is a high degree of signal processing in the olfactory bulb and the olfactory cortex of the brain. The odour classes demonstrated during an experiment of this type may well have been due to interpretation of complex signals by the brain and olfactory bulb which bear no relationship to the interaction of the acids with specific receptors. The fact that our sense of smell remains constant during the receptor neurone turnover indicates the presence of a complex processing system. The evidence of specific anosmia towards iso-valeric acid (Amoore, 1970) and the recent biochemical evidence that there is a receptor present (Shirley *et al*, 1987b,c) which seems to be relatively specific for that size of acid does seem to suggest that studies of this type may well have some relevance.

When the olfactory receptors have been extracted, purified and investigated using biochemical means it should be possible to compare the results of psychophysical studies, such as these, with the binding specificity of the purified receptors. Only then will we know whether the odour classes identified by the various psychophysical techniques correlate with specific receptors or whether the classes are a consequence of processing by the brain or the olfactory bulb.

OVERALL DISCUSSION.

The demonstration of an odorant-stimulated adenylate cyclase in chapter two of this thesis, together with similar evidence from other investigators, lends support to the hypothesis that cyclic AMP is a second messenger in olfaction. The presence of large amounts of olfactory cyclic nucleotide phosphodiesterases, shown in chapter three, also supports this hypothesis. The inability of many odorants, however, to stimulate adenylate cyclase may be indicative of either an alternative transduction mechanism, or an artifact of the in-vitro system. A potential candidate for the former possibility would be an odorant-stimulated cyclic nucleotide phosphodiesterase. However, no evidence for this transduction mechanism could be found and identification of any alternative transduction mechanism remains elusive. The ability of calcium to inhibit olfactory adenylate cyclase, and to activate cyclic nucleotide phosphodiesterase, suggests that a rise in intracellular calcium may be a mechanism for desensitisation.

It seems likely, therefore, that binding of an odorant to a receptor on the cilia leads to activation of a G-protein; this then activates adenylate cyclase which catalyses the production of cyclic AMP. The raised intracellular cyclic AMP concentration can activate protein kinases, which phosphorylate ion-channels in the cilia. This causes depolarisation and ultimately, an action potential in the neurone. Alternatively, cyclic AMP could activate the ion-channels directly as in vision. Spontaneous inactivation of the G-protein by GTP hydrolysis would stop the activation of adenylate cyclase. The action of cyclic nucleotide

phosphodiesterases would then return the concentration of cyclic AMP back to resting levels. The depolarisation of the ciliai membrane could activate voltage gated calcium ionophores resulting in an influx of calcium. This would help to return the system back to initial conditions by activating the phosphodiesterase and inhibiting the cyclase. The odorant-stimulated adenylate cyclase could serve as a useful 'handle' for receptor purification.

Electrophysiological evidence, summarised in the first chapter, seems to indicate that olfactory neurones have a broad specificity towards odorants. This broad specificity, coupled with the hydrophobic nature of most odorants, would explain the lack of success with the identification of receptors by ligand binding experiments. The structure of the receptors, presumed to be proteinaceous, has therefore to be inferred from electrophysiological and psychophysical studies. Chapter four of this thesis is a psychophysical study of a series of fatty acid odorants. A homologous series of odorants is a desirable feature of such a study as it will reduce much of the complexity of the total system. The threshold and profiling results seem to suggest the presence of at least four receptors involved in fatty acid odour reception. Classic structure-activity methods failed, presumably due to the multiple receptors involved. The significance of the results from this type of study cannot be judged until the purified receptors can be studied by biochemical means.

APPENDIX 1: SAS procedures used for ANOVA of the profiling results.

In the actual SAS file, the database would be contained between the 'cards;' and the ';' commands and is given in appendix 5. The second line codes the fields in the database and can be used to decode the database in appendix 5.

```
DATA OLF;
  INPUT SESSION 4-6 ASSESSOR 7-9 ACID 12-13 ORDER 14 @20(V1-V25)(2.0);
  TITLE 'SAS ANALYSIS OF FATTY ACID PROFILING DATA';
  LABEL V1='INITIAL IMPACT'
        V2='VINEGAR'
        V3='CARAMEL'
        V4='MOTHBALLS'
        V5='SHARP'
        V6='STILTON'
        V7='CHEDDAR'
        V8='SWEATY SOCKS'
        V9='AMMONIA'
        V10='NAPPIES'
        V11='WET CARDBOARD'
        V12='MUSTY'
        V13='MOULDY'
        V14='VINYL'
        V15='PEARDROPS'
        V16='BOILED SWEETS'
        V17='APPLE'
        V18='COCONUT'
        V19='FATTY/CANDLES'
        V20='FATTY/TWEEN'
        V21='SOAPY'
        V22='FLASH LIQUID'
        V23='TOILET BLOCK'
        V24='OVERALL INTENSITY'
        V25='PERSISTANCE';
  CARDS;
;
PROC SORT DATA=OLF;
   BY ACID;
PROC PRINT;
PROC MEANS;
   VAR V1-V25;
   BY ACID;
PROC GLM;
   CLASS ASSESSOR ACID;
   MODEL V1-V25=ACID ASSESSOR ACID*ASSESSOR;
   MEANS ACID/DUNCAN;
ENDSAS;
```

APPENDIX 2: SAS procedures used for the PCA of the profiling results.

The second line allows decoding of the database found in appendix 5, as with appendix 1.

```
DATA OLF;
  INPUT SESSION 4-6 ASSESSOR 7-9 ACID 12-13 ORDER 14 @20(V1-V25)(2.0);
  TITLE 'SAS ANALYSIS OF FATTY ACID PROFILING DATA';
  LABEL V1='INITIAL IMPACT'
        V2='VINEGAR'
        V3='CARAMEL'
        V4='MOTHBALLS'
        V5='SHARP'
        V6='STILTON'
        V7='CHEDDAR'
        V8='SWEATY SOCKS'
        V9='AMMONIA'
        V10='NAPPIES'
        V11='WET CARDBOARD'
        V12='MUSTY'
        V13='MOULDY'
        V14='VINYL'
        V15='PEARDROPS'
        V16='BOILED SWEETS'
        V17='APPLE'
        V18='COCONUT'
        V19='FATTY/CANDLES'
        V20='FATTY/TWEEN'
        V21='SOAPY'
        V22='FLASH LIQUID'
        V23='TOILET BLOCKS'
        V24='OVERALL INTENSITY'
        V25='PERSISTANCE';
  CARDS;
;
PROC SORT DATA=OLF;
   BY ACID;
PROC MEANS;
   VAR V1-V25;
   BY ACID;
   OUTPUT OUT=PCADATA MEAN=V1-V25;
PROC FACTOR METHOD=PRINCIPAL DATA=PCADATA SCREE SCORE ROTATE=VARIMAX
PREPLOT PLOT NFACTOR=3 CORR RES OUTSTAT=PCARES;
    VAR V2 V4 V5 V6 V7 V8 V12 V13 V14 V16;
PROC SCORE DATA=PCADATA SCORE=PCARES OUT=SCOREDAT;
PROC PRINT DATA=SCOREDAT;
PROC PLOT;
    PLOT FACTOR2*FACTOR1=ACID;
    TITLE2 'PLOT OF THE FIRST TWO PRINCIPAL COMPONENTS';
PROC PLOT;
    PLOT FACTOR3*FACTOR1=ACID;
    TITLE2 'PLOT OF THE FIRST AND THIRD PRINCIPAL COMPONENTS';
PROC PLOT;
    PLOT FACTOR3*FACTOR2=ACID;
    TITLE2 'PLOT OF THE SECOND AND THIRD PRINCIPAL COMPONENTS';
ENDSAS;
```

APPENDIX 3: SAS procedures used for regression analysis of the threshold data.

The physico-chemical parameters used for the analysis are contained in this listing. The fields of the database can be decoded by reference to the second command line. The abbreviations are as follows: (MWT) molecular weight, (PKA) PK_a, (DIAM) diamagnetic resonance, (BP) boiling point, (VP) vapour pressure, (MP) melting point, (GC) retention time at 180°C on an FFAP gas chromatography column, (DENS) density, (REF) refractive index, (LTHRESH) logarithm of determined threshold, (THRESH) determined threshold. The parameters were obtained from the 1987 Aldrich catalogue, The Handbook of Chemistry and Physics (45th edition) or were determined.

```
 DATA THRESH;
      INPUT ACID $ 1-13 MWT 11-15 PKA 16-19 DIAM 21-25 BP 28-32 VP 34-38
MP 40-44 GC 46-49 DENS 52-57 REF 58-63 LTHRESH 65-68 THRESH 70-73;
      TITLE 'SAS ANALYSIS OF FATTY ACID THRESHOLD DATA';
      CARDS;
FORMIC     46.0 3.75 19.98   100.5 -20.0 8.4    1.9    1.220 1.3714 3.78 6000
ACETIC     60.1 4.76 31.54   118.2 -17.2 16.7   2.75   1.049 1.3721 2.66 460
PROPIONIC 74.1 4.88 43.50   141.1 4.6    -21.5 3.57   0.999 1.3874 2.81 640
BUTYRIC    88.1 4.82 55.16   163.5 25.5   -7.9  4.75   0.959 1.3991 0.58 3.8
VALERIC   102.14.86 66.85   186.5 42.2   -34.5 6.90   0.939 1.4086 0.92 8.4
HEXANOIC  116.24.87 78.55   203.0 71.4   -3.0  10.0   0.92741.4163 1.63 43
HEPTANOIC 130.24.89 88.60   223.0 78.0   -8.8  14.7   0.91851.4216 1.66 46
OCTANOIC  144.24.90 101.6   239.7 92.3   16.7  21.25 0.910 1.4280 1.15 14
;
PROC RSQUARE DATA=THRESH OUTEST=EST
             MSE JP GMSEP CP AIC BIC SBC R SELECT=1;
 MODEL LTHRESH=MWT PKA DIAM BP VP MP GC DENS REF;
PROC PRINT DATA=EST;
ENDSAS;
```

APPENDIX 4: SAS procedures used for regression analysis of the profiling data.

The physico-chemical parameters used for this analysis are the same as in appendix 3. The database for this analysis differs in that the scores for the first three principal components, for each acid, are included (PC1, PC2 and PC3).

```
DATA THRESH;
     INPUT ACID 1 MWT 3-6 PKA 8-11 DIAM 13-17 BP 19-23 VP 25-29
MP 30-34 GC 35-39 DENS 40-45 REF 46-50 LTHRESH 52-55 PC1 57-61
PC2 63-67 PC3 69-73;
     TITLE 'SAS CORRELATION ANALYSIS';
     CARDS;
1 46.0 3.75 19.98 100.5 -20.08.4   1.9  1.220 1.371 3.78 -1.09 -1.13 -0.57
2 60.1 4.76 31.54 118.2 -17.216.7 2.75 1.049 1.372 2.66 -0.95 -1.07 -0.68
3 74.1 4.88 43.50 141.1 4.6   -21.53.57 0.999 1.387 2.81 -0.93 -0.73 -0.57
4 88.1 4.82 55.16 163.5 25.5 -7.9 4.75 0.959 1.400 0.58 -0.43 -0.99 -0.18
5 102  4.86 66.85 186.5 42.2 -34.56.90 0.939 1.409 0.92  0.02  1.46 -0.58
6 116  4.87 78.55 203.0 71.4 -3.0 10.0 0.92741.422 1.63  1.63 -0.09 -0.71
7 130  4.89 88.60 223.0 78.0 -8.8 14.7 0.91851.422 1.66  1.10 -0.69  0.12
8 144  4.90 101.6 239.7 92.3 16.7 21.250.910 1.428 1.15  1.41 -0.33 -0.50
;
PROC RSQUARE DATA=THRESH OUTEST=EST MSE JP GMSEP CP
                         AIC BIC SBC B SELECT=1;
    MODEL PC1=MWT PKA DIAM BP VP MP GC DENS REF LTHRESH;
    MODEL PC2=MWT PKA DIAM BP VP MP GC DENS REF LTHRESH;
    MODEL PC3=MWT PKA DIAM BP VP MP GC DENS REF LTHRESH;
PROC PRINT DATA=EST;
ENDSAS;
```

APPENDIX 5: Raw profiling data used for SAS analysis.

Each horizontal line is a single determination by an assessor. The rows can be decoded by reference to appendix 1 or 2 and are as follows: session, assessor, acid (coded 1 to 11), order and finally the scores for all twenty five descriptors.

```
CARDS;
  1  1  111    4 2 3 2 1 1 1 1 1 1 1 3 1 6 9 1 3 1 1 1 1 1 4 5 2
  1  1   62    8 3 1 1 4 1 1 1 5 5 1 1 3 7 3 1 1 1 1 1 1 1 3 5 2
  1  1   43    3 1 1 2 5 1 4 1 2 3 1 1 1 3 4 2 2 1 1 1 4 1 1 3 2
  1  1   54    3 2 1 1 2 1 4 6 2 3 1 1 1 3 3 1 1 1 1 2 1 1 1 2 3
  1  2   41    2 2 1 1 2 7 4 6 1 1 1 2 2 1 1 1 1 1 2 2 2 1 1 7 3
  1  2   92    2 2 2 1 2 8 9 7 1 1 1 2 2 1 1 1 1 1 2 2 1 1 1 8 4
  1  2   73    2 2 1 1 2 2 2 2 1 6 1 2 2 1 1 1 1 1 1 2 2 1 1 4 3
  1  2   14    6 2 2 1 2 3 3 1 8 5 1 2 2 1 1 1 1 2 1 1 1 1 1 5 4
  1  3   11    8 8 1 1 7 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 7 7
  1  3  112    8 3 1 1 4 1 1 8 5 5 1 1 1 1 1 7 1 1 1 1 1 1 1 9 8
  1  3   63    8 1 1 5 1 1 1 3 7 1 5 8 1 6 1 4 1 1 4 1 1 1 1 8 8
  1  3   24    8 8 1 1 5 1 1 1 1 1 1 1 1 1 1 3 7 1 1 1 1 1 1 7 7
  1  4   31    8 7 1 3 7 4 6 3 2 1 1 1 4 2 1 1 1 1 1 1 1 2 1 8 7
  1  4   62    7 3 2 4 8 1 1 2 4 1 2 3 1 6 1 1 1 2 8 5 3 3 2 8 7
  1  4   23    7 6 1 1 6 1 1 1 6 1 1 1 1 3 1 1 1 1 1 1 1 3 1 6 6
  1  4   44   10 4 1 2 7 5 8 4 7 1 1 1 8 3 1 1 1 1 1 1 1 1 1 9 8
  1  5   61    8 3 1 7 1 1 1 1 1 1 1 5 3 3 1 1 1 1 1 1 1 1 1 5 3
  1  5   22    9 9 1 1 7 1 1 1 4 1 1 1 1 1 1 1 1 1 1 1 3 1 3 8 4
  1  5   83    9 1 1 1 1 1 1 1 1 1 1 1 1 4 1 1 1 1 4 2 9 1 1 8 4
  1  5   94    7 3 1 1 2 7 5 3 1 1 1 1 3 1 1 1 3 1 1 1 1 1 1 7 7
  1  6   71    8 6 6 2 1 3 3 5 1 6 3 3 310 3 5 7 7 6 8 4 3 5 8 5
  1  6   32    9 9 3 5 9 1 1 7 9 7 3 1 1 1 7 1 7 5 1 1 1 4 410 8
  1  6   93    5 7 7 5 7 1 1 6 8 7 5 5 6 7 4 4 8 4 3 6 7 3 7 9 3
  1  6  114    9 3 9 1 3 8 8 8 4 9 9 710 5 8 8 6 9 9 2 6 8 7 6 9
  1  7   61    7 1 1 1 1 3 1 4 2 1 1 4 1 5 1 2 1 1 1 1 1 1 2 4 5
  1  7   72    6 2 1 1 1 1 1 1 1 1 1 3 1 8 1 1 1 1 1 1 1 1 2 7 4
  1  7   63    7 1 1 1 1 5 7 1 2 1 1 1 6 2 1 1 1 1 1 1 1 1 2 7 4
  1  7   84    3 2 1 3 3 4 2 1 5 1 3 5 1 3 1 1 1 1 1 1 1 1 1 3 7
  1  8  101    8 3 1 1 3 1 6 2 1 3 1 1 5 2 2 1 2 1 1 1 1 2 1 9 7
  1  8   12    6 4 2 1 7 1 3 2 1 4 1 1 4 1 1 1 2 1 1 1 1 1 1 7 7
  1  8   33    5 6 1 1 6 1 3 2 1 4 1 1 4 1 1 1 2 1 1 1 1 1 1 6 5
  1  8   64    7 1 1 1 2 1 1 2 1 2 1 3 3 4 1 1 1 1 2 1 1 1 1 8 6
  1  9   51    4 3 1 1 1 5 7 7 1 1 1 1 6 1 1 1 1 1 1 4 1 1 1 3 3
  1  9   82    3 3 1 5 5 1 1 1 3 5 1 7 1 1 1 1 1 1 1 1 1 5 5 3 3
  1  9   13    8 8 1 1 8 1 1 1 3 1 1 1 1 1 1 1 1 1 1 1 1 6 1 8 7
  1  9   34    8 8 1 1 9 1 1 1 4 1 1 7 4 1 1 1 1 1 1 1 1 1 1 9 8
  1 10   21    910 1 110 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 110 2
  1 10  102   10 1 1 1 1 3 4 4 2 2 1 1 1 1 1 1 1 1 1 1 1 1 1 5 3
  1 10   53    3 1 1 1 110 2 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 4 2
  1 10   74    9 3 1 1 2 1 1 1 1 1 1 1 1 3 1 1 1 1 1 1 1 1 1 4 1
  1 11   91    8 1 1 1 3 8 9 6 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 7 2
  1 11   52    5 1 1 1 3 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 9 8 4 6
  1 11  103    3 1 1 1 1 1 1 1 1 1 1 1 1 1 2 8 3 1 1 1 1 1 1 3 2
  1 11   64    7 1 1 4 2 1 1 1 1 1 1 1 1 9 1 1 1 1 1 1 1 5 2 9 3
  1 12   81    9 5 1 3 6 2 1 3 2 2 1 3 4 4 1 1 1 1 2 1 1 1 2 5 4
  1 12   42   1010 1 2 4 2 2 1 3 2 1 2 5 1 1 1 1 1 1 2 1 1 210 8
  1 12  113    3 1 3 1 1 1 1 3 2 1 1 2 1 1 2 5 2 1 1 2 1 1 3 6 6
  1 12  104    7 1 2 1 1 1 1 2 1 1 1 1 1 1 6 9 2 2 1 1 1 1 2 5 6
  2  1   61    3 2 2 3 5 1 1 1 1 1 1 5 1 7 1 1 1 1 1 1 1 1 1 5 3
  2  1  102    5 1 1 2 2 1 1 1 2 2 1 1 1 4 7 1 5 1 1 1 1 1 1 3 2
  2  1  113    3 1 1 1 1 1 1 1 2 1 1 1 1 4 4 1 1 1 1 1 1 1 1 2 1
  2  1   14    3 6 1 2 8 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 110 6
  2  2   51    8 2 2 1 7 8 8 8 1 7 1 2 2 1 1 1 1 1 1 1 1 1 1 8 7
  2  2   22    7 9 1 110 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 11010
  2  2   43    9 4 2 1 8 6 6 5 2 3 2 2 2 1 2 2 1 2 2 2 1 1 1 9 5
  2  2  114    8 1 2 8 3 4 3 3 6 4 2 2 2 8 6 6 1 1 1 1 2 1 2 7 9
  2  3   91    8 6 1 1 5 1 6 8 5 1 1 1 1 1 1 5 1 1 1 1 1 1 1 8 9
  2  3  112    7 3 3 1 3 1 1 6 5 1 1 1 1 1 7 7 1 1 1 1 1 1 1 7 5
  2  3   33    9 9 1 1 6 1 1 1 1 1 1 1 1 1 1 7 1 1 1 1 1 1 1 9 8
  2  3   84    3 1 1 8 2 1 1 1 4 1 1 8 1 1 1 3 1 1 5 1 1 1 1 6 6
  2  4   21    9 7 3 6 8 2 1 1 4 2 1 2 2 2 1 1 3 1 1 1 1 2 1 8 6
  2  4   12    9 8 2 4 9 1 1 1 4 1 1 2 2 5 1 1 1 1 3 1 1 2 2 9 7
  2  4   63    9 5 2 7 7 1 1 1 5 1 1 2 3 6 1 1 1 1 5 2 2 1 2 9 6
  2  4   94    8 6 1 3 8 4 8 3 5 1 1 2 4 2 1 1 1 1 3 1 1 1 1 9 6
  2  5   71    9 5 1 2 7 1 1 1 3 1 1 3 1 1 1 1 1 1 1 1 2 1 1 9 7
```

2	5	42	8	1	1	1	1	9	8	4	1	1	1	1	2	2	1	1	1	1	1	1	1	1	1	8	5
2	5	93	2	1	1	1	1	3	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1
2	5	64	2	1	1	4	1	1	1	1	2	1	1	2	2	2	1	1	1	1	1	1	1	1	1	2	1
2	6	111	9	7	9	8	6	1	1	8	8	8	5	5	4	8	1	5	6	3	7	5	3	1	1	10	6
2	6	52	10	8	1	9	7	1	1	4	9	6	1	1	4	7	1	1	1	3	1	1	1	1	2	9	6
2	6	73	7	4	6	1	8	1	1	7	4	4	1	8	5	10	7	1	4	1	1	7	3	1	3	8	6
2	6	64	7	1	6	5	8	1	1	7	6	8	5	5	7	9	5	1	4	3	8	1	3	3	6	8	6
2	7	11	8	6	1	1	8	1	1	1	6	1	1	1	1	1	1	4	2	1	1	1	1	1	1	7	7
2	7	62	5	2	1	1	4	5	7	3	2	2	1	1	6	2	1	1	1	1	1	1	1	1	2	4	6
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2	7	54	6	3	1	1	1	3	3	6	3	1	1	3	2	3	1	2	5	1	1	1	1	1	1	6	5
2	8	31	8	5	2	1	6	1	3	2	1	3	1	1	3	1	1	1	2	1	1	1	1	2	2	9	7
2	8	92	7	1	2	1	3	2	7	4	1	2	1	1	5	1	1	1	2	1	1	1	1	1	1	7	7
2	8	53	7	2	1	1	3	3	6	3	1	2	1	1	3	2	1	1	2	1	1	1	1	2	1	8	8
2	8	104	6	2	1	1	5	1	1	1	1	2	1	2	3	3	3	5	3	1	1	1	1	2	1	8	7
2	9	101	7	7	1	1	5	5	6	6	1	1	1	1	1	1	1	5	5	1	1	1	1	1	1	6	5
2	9	82	8	8	1	1	8	1	1	1	2	1	3	6	5	1	1	1	1	1	1	1	1	1	7	8	6
2	9	23	8	9	1	1	7	1	1	1	1	1	1	6	1	1	1	1	6	1	1	4	1	7	1	8	8
2	9	74	8	2	1	5	7	1	1	7	1	1	7	7	7	7	1	1	1	1	6	1	6	1	1	8	7
2	10	41	10	4	1	1	3	6	5	6	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	7	1
2	10	72	7	2	1	1	3	2	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	4	1
2	10	13	2	9	1	1	9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	10	2
2	10	34	5	6	2	1	4	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	5	1
2	11	81	6	1	1	1	2	1	1	1	1	1	1	1	1	8	1	1	1	1	1	1	1	1	1	5	3
2	11	62	8	1	1	1	3	1	1	1	1	1	1	1	1	8	1	1	1	1	1	1	1	1	1	7	3
2	11	103	2	1	1	1	1	1	1	1	1	1	1	1	1	1	3	7	3	1	1	1	1	1	1	6	2
2	11	44	2	1	1	1	1	1	1	1	1	1	1	1	1	1	2	5	2	1	1	1	1	1	1	4	2
2	12	61	9	3	1	3	7	1	1	3	2	3	1	2	3	1	1	1	1	1	3	4	1	1	2	6	4
2	12	32	4	6	2	2	3	1	2	1	2	1	1	1	1	1	1	1	1	1	2	3	1	1	2	8	9
2	12	63	3	1	2	2	2	1	1	2	2	2	1	1	3	3	1	1	1	1	2	4	1	1	2	8	6
2	12	24	1	1	1	1	2	1	1	2	2	1	1	1	1	1	1	1	1	1	2	1	1	1	1	2	1
3	1	101	4	2	1	2	3	1	1	1	2	2	1	1	1	4	6	1	4	2	1	1	3	1	1	4	2
3	1	92	3	2	1	1	2	1	4	6	2	3	1	1	1	3	3	1	1	1	1	2	1	1	1	2	3
3	1	113	7	1	1	3	2	1	1	1	2	1	1	1	1	2	6	1	2	1	1	1	2	1	1	6	1
3	1	14	3	7	1	1	9	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1	9	6
3	2	91	8	7	2	1	6	8	8	7	1	5	1	2	2	2	1	1	1	1	1	1	2	1	1	8	7
3	2	42	4	8	2	1	3	5	5	3	1	4	2	2	3	2	1	1	1	2	1	2	2	1	1	7	6
3	2	73	5	4	1	1	2	1	1	1	1	2	2	2	2	1	1	1	1	3	1	2	2	1	1	2	2
3	2	84	9	1	1	2	3	1	1	1	8	2	2	2	2	7	3	1	1	1	1	2	2	1	1	7	6
3	3	61	7	1	1	6	1	1	1	3	3	1	1	8	1	1	1	3	1	1	1	1	1	1	1	7	6
3	3	22	8	8	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	7	6
3	3	83	6	1	1	5	1	1	1	1	1	1	1	7	1	1	1	1	1	1	4	1	1	1	1	6	6
3	3	104	8	6	1	1	1	1	1	7	4	1	1	1	1	1	1	8	1	1	1	1	1	1	1	9	8
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6	11	114	8	1	1	1	1	1	1	1	1	1	1	1	1	1	4	9	7	1	1	1	1	1	1	7	3
6	12	71	4	1	1	2	1	1	1	1	2	2	2	3	4	6	1	1	1	1	3	4	1	1	1	6	3
6	12	82	5	1	1	3	2	1	2	2	3	4	3	4	2	3	1	1	1	3	5	5	5	1	1	6	4
6	12	63	5	1	1	3	1	2	2	2	3	4	2	2	2	7	1	1	1	2	3	3	3	1	1	7	1
6	12	14	8	7	3	1	8	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	8	1
6	13	61	7	4	1	6	5	1	1	1	3	5	1	5	3	4	3	1	1	1	1	1	3	1	5	8	3
6	13	42	3	1	1	1	1	3	5	5	2	6	1	4	1	3	1	1	1	1	1	3	2	1	1	4	3
6	13	63	3	1	1	3	2	2	1	1	4	1	1	5	4	6	1	1	1	1	1	1	3	1	3	5	4
6	13	24	8	8	1	1	6	1	1	1	3	1	1	1	1	3	1	1	3	1	1	1	1	1	1	7	6

APPENDIX 6

Biochem. J. (1986) 240, 865–867 (Printed in Great Britain)

Olfactory adenylate cyclase of the rat

Stimulation by odorants and inhibition by Ca^{2+}

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Membranes prepared from the olfactory mucosa of the rat show a high level of adenylate cyclase activity. The activity increases up to 2-fold in the presence of physiologically relevant concentrations of odorants and is inhibited by Ca^{2+}. The level of cyclase activity found is sufficient to explain the speed of olfactory transduction, which occurs on a time scale of tens of milliseconds.

INTRODUCTION

The possible involvement of cyclic AMP in olfactory transduction has long been suspected. Kurihara & Koyama (1972) demonstrated the presence of high levels of adenylate cyclase in the olfactory mucosa, but did not report any attempts to stimulate this activity with odorants. Menevse *et al.* (1977) showed that cyclic AMP analogues and phosphodiesterase inhibitors affected the production of the EOG (electro-olfactogram) (an indicator of early electrical events in odour transduction).

Menevse *et al.* (1977) found that odorants did not stimulate the adenylate cyclase of olfactory membrane preparations. However, details of their preparation were not given. In the present paper we report investigations of sonicated olfactory material from the rat which show the presence of an odour-stimulated adenylate cyclase.

Recently, an odour-modulated adenylate cyclase has been found in the frog (Pace *et al.*, 1985; Ashoh, 1986). This seems to have properties very similar to the rat tissue preparation described here.

MATERIALS AND METHODS

Animals

Male Wistar rats of about 300 g body weight were used.

Chemicals

The radiolabelled biochemicals were supplied by Amersham International; all other biochemicals were from Sigma Chemicals, except isoamole, were of analytical quality. Odorants were of the highest commercially available quality and were used without further purification.

Adenylate cyclase activity assay

We followed the procedure of White & Karr (1978) with minor modifications. Our 80 μl incubation volume contained phosphate buffer (50 mM), cyclic AMP (3 mM), isobutylmethylxanthine (1.6 mM), phosphocreatine (18 mM), creatine phosphokinase (8.5 units), GTP (0.01 mM), $MgCl_2$ (2.3 mM), AMP (1 mM; approx. 300 nCi of [α-^{32}P]AMP), EGTA (0.12 mM) and bovine serum albumin (0.08 mg). The 40 min incubations (30 °C) were terminated by the addition of 0.15 ml of 1 M-$HClO_4$. Separation and counting of the labelled product followed the method of White & Karr (1978).

Protein assay

Protein was assayed by the method of Hartree (1972), with bovine serum albumin as standard. [The concentration as measured by this assay is consistently two or three times higher than that measured by the Bio-Rad protein assay, which is based on the method of Bradford (1976).]

Calcium buffer

Where a calcium buffer was used, the active ingredients were phosphate buffer (50 mM, pH 7.5 at 30 °C), EGTA (0.12 mM), *N*-hydroxyethylenediaminetriacetic acid (0.75 mM), ATP (1 mM) and $MgCl_2$ (2.3 mM). The stability constants were taken from Sillén & Martell (1964, 1971) and Martell & Smith (1974), and corrected, if necessary, to 30 °C.

Preparation of crude olfactory adenylate cyclase

Medium A was NaCl (0.9% w/v)/EGTA (1 mM)/phosphate buffer (5 mM), pH 7.0; medium B was NaCl (0.9% w/v)/phosphate buffer (5 mM), pH 7.0.

The following preparation was designed to minimize the use of organic chemicals, in case substances like (protective) mercaptans, organic buffers, proteolytic inhibitors and sucrose stimulate the olfactory receptors. Results suggest, however, that not all of these components interfere and that the preparation can be achieved by using rather more conventional buffer systems.

The ethmoturbinates were removed from a freshly killed rat and soaked in cold medium A to remove superficial blood and debris. The solution was changed three times at 3 min intervals with minimal agitation. The tissue was then sonicated in 10 vol. of medium B. Sonication was for 5 s in ice at the medium power setting of the MSE 100 W disintegrator using an exponentially tapered probe of 3 mm tip diameter. The maximum amount of material sonicated in any one tube was that derived from two rats.

The suspension was removed and the tissue rinsed with a further 10 vol. of medium B. The extracts were pooled and centrifuged at 1000 *g* for 30 min at 4 °C. The supernatant was withdrawn and re-centrifuged at 20000 *g* for 40 min at 4 °C. This pellet was resuspended

Abbreviation used: EOG, electro-olfactogram.

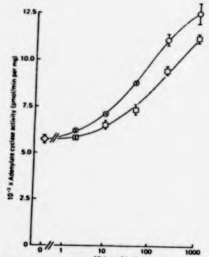

Fig. 1. Variation of adenylate cyclase activity with the concentration of added odorant

○, Carvone; □, acetophenone. The error bars indicate the S.E.M. of triplicate determinations on a single preparation.

in medium B, usually 2 vol. based on the original weight of tissue. Yields were typically 0.4–0.6 mg of protein per rat.

RESULTS

Adenylate cyclase activity (typically 500 pmol/min per mg of protein) was found in the olfactory preparations. Cyclic AMP production, under the conditions of the assay, was linear with time for up to 1 h.

Preparations showed enhanced cyclase activity when assayed in the presence of odorants (Fig. 1), and activity varied with the concentration of added odorant. The concentration range of the odorants shown in Fig. 1 is physiologically relevant, since the vapour pressures over the assayed solutions [calculated from the water/air partition coefficients (S. G. Shirley, E. H. Polak, D. A. Edwards, M. A. Wood & G. H. Dodd, unpublished work)] are about 0.003–3 Pa for carvone and 0.008–8 Pa for acetophenone.

The EOG is an indicator of the early electrical events in odour transduction (Ottoson, 1970). The variation of its amplitude with odorant concentration can be described by the equation:

$$\log A = m \times \log C + \text{constant} \qquad (1)$$

(Ottoson, 1956; S. G. Shirley, E. H. Polak, D. A. Edwards, M. A. Wood & G. H. Dodd, unpublished work)

where C is the concentration of the odorant. For most odorants the coefficient m is in the range 0.3–0.5. The data in Fig. 1 also fit this equation. In this case A is the cyclase activity minus the activity with no added odorant. For carvone, m is 0.34 and the correlation coefficient (r) is 0.97; for acetophenone m is 0.43 and r is 0.99.

Ca^{2+}, at concentrations in and above the physiological range, inhibited the cyclase activity, in both the presence and the absence of odorant (Fig. 2).

Other odorants also stimulated the activity of olfactory preparations. At 1 mM, (+)-carvone produced nearly the same stimulation as the (−)-isomer. Isopentyl acetate and 1–8-cineole at 1 mM produced stimulations of 50 and 35% of the resting activity respectively. Hexanol and triethylamine at 3 mM gave stimulations of 61 and 35% respectively. (+)-Camphor stimulated the activity by 34% at half-saturated concentration.

We used glycerol as an example of a non-odorous organic material. This failed to stimulate the olfactory cyclase when it was applied at 30 mM.

Sucrose (a common component of membrane preparation media) at 270 mM in the assay mixture caused a doubling of the activity, and odorants added in the

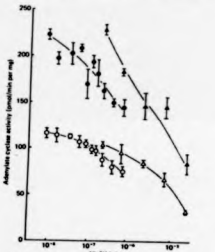

Fig. 2. Ca^{2+} inhibits both odour-stimulated and basal adenylate cyclase activity

The left-hand curves were measured in the presence of the calcium buffer described in the Materials and methods section. For the right-hand curves (different preparations) the 10 mM-phosphate buffer provided the calcium-buffering capacity. The error bars represent the S.E.M. of triplicate determinations. ○, △, No added odour; ▲, 1 mM-carvone; ●, 2 mM-acetophenone.

presence of sucrose caused no further stimulation. The above results are based on duplicate determinations whose variability was 4% (s.e.).

No stimulation of the cyclase was observed in the presence of either 1 mM-histamine or 1 mM-isoprenaline. NaF in the assay mixture at 10 mM caused a 2.5-fold increase in cyclase activity. Guanosine 5′-O-(3-thiotriphosphate) and guanosine, 5′-O-[(γ-imido)triphosphate, each at 0.1 mM, caused 3.8- and 2.8-fold increases respectively. Guanosine 5′-O-(2-thiodiphosphate) at 0.01 mM decreased the activity of the cyclase preparation to 20% of the initial level.

DISCUSSION

Pace *et al.* (1985) and Anholt (1986) have suggested tht olfaction in the frog is mediated by a cyclic AMP system. This may be true for the rat also; we have found an adenylate cyclase responding to physiologically relevant concentrations of odorants in a way which parallels the early electrical events of odour transduction. The effects of the guanine nucleotide analogues on the rat cyclase indicate the possible involvement of a G-protein, as has been demonstrated for the frog by Pace *et al.* (1985). An inhibition of the frog cyclase by Ca^{2+} has been shown by Anholt (1986).

Menevse *et al.* (1977) found that odorants did not stimulate the cyclase activity of olfactory membranes. These membranes were prepared in sucrose, which we have found to stimulate the cyclase and may have masked any response to odour.

Receptor heterogeneity (Polak, 1973) could explain the shape of the enzyme-activity-versus-odorant-concentration curve (Pace *et al.*, 1985). Some recent results (S. G. Shirley, E. H. Polak, D. A. Edwards, M. A. Wood & G. H. Dodd, unpublished work) on the variation of EOG amplitude with odorant concentration support this view.

In general, adenylate cyclase systems are compartmentalized within the cell (Earp & Steiner, 1978). The olfactory transduction mechanism is believed to reside in the cilia and terminal swelling of the olfactory primary cells (Getchell *et al.*, 1984). The high levels of cyclase activity which we have observed would, if confined to this region, be sufficient to explain the speed of response of the olfactory system. Our data, combined with the ultrastructural data of Menco (1980), would imply cyclase activities of about 10^{-16} mol/s per cell (minimum). The combined volume of terminal swelling and cilia is 5×10^{-10} l/cell (Menco, 1977). With a doubling of activity on stimulation, the local cyclic AMP concentration should rise with an initial rate of some 200 μM/s. Changes in cyclic AMP concentrations of the order of 1 μM are of physiological significance. [Terasaki & Brooker (1977) estimated that half-maximal binding of cyclic AMP in rat heart occurred at free concentrations of about 1 μM.] The response time of the system should therefore be in the millisecond range.

The olfactory adenylate cyclase should be a powerful tool for the study of the olfactory receptors *in vitro*, providing a monitor for the solubilization and purification of the receptors.

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APPENDIX 7

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Binding of aromatic isonitriles to haemoglobin and myoglobin

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A series of aromatic isonitriles were synthesized and their binding to sheep haemoglobin and horse-heart myoglobin was investigated. The disubstituted ligands 2,6-dimethylphenylisonitrile and 2,6-diethylphenylisonitrile were found to bind to horse-heart myoglobin with affinities ranging from 500 to 5000 times greater than that of ethylisonitrile (4.6×10^{-8} M) which has been the tightest binding isonitrile ligand for myoglobin thus far reported. The tight binding was not found to vary significantly with pH or temperature. An explanation for the unexpectedly high affinity is offered in terms of the electronic structure of aromatic isonitriles.

INTRODUCTION

Isonitriles have been suggested as suitable probes for the haemoglobin binding site since 1951 (St. George & Pauling, 1951). St. George and Pauling examined the binding of ethylisonitrile, isopropylisonitrile and *tert*-butylisonitriles to both haemoglobin and a model system comprising ferrihaem chloride in aqueous solution. The affinity of the model haem system for the isonitriles was effectively constant for all the ligands. From this it was concluded that the strength of the iron–isonitrile bond was constant regardless of the aliphatic group and hence, any variation in affinity of the protein for the ligand can be attributed solely to interactions between the ligand and amino acid residues surrounding the distal site of the protein. Such effects are termed 'distal effects'.

However, the observation that the affinities of bovine haemoglobin for ethylisonitrile, isopropylisonitrile and *tert*-butylisonitrile varied greatly (K_d values 0.1 mM, 0.3 mM and 22.0 mM respectively) supported Pauling's proposal that the haem group was buried within the haemoglobin molecule and that incoming ligands were subject to steric interaction.

Reisberg & Olson (1980) in a study using a series of 13 aliphatic isonitrile probes estimated the favourable hydrophobic contribution for ligand binding to haemoglobin by calculating the chemical potential of ligands in aqueous solution assuming the chemical potential of the same ligand in the organic phase to be zero. This was achieved using the method of Harris *et al.* (1973) which demonstrates a linear relationship between the surface area of a molecule and the free energy of partition from aqueous to organic phase. In this way Reisberg & Olson transformed the data from simple equilibrium binding to component factors of favourable hydrophobic interaction and unfavourable steric hindrance and so were able to compile a free-energy steric map of the sixth coordinate site of haemoglobin.

To our knowledge, the binding of aromatic isonitriles to haemoglobin or myoglobin has not been reported in the literature. We have synthesized a series of substituted aromatic isonitriles. The 2,6-disubstituted aromatic isonitriles are more stable than the monosubstituted ligands (i.e. they show neither heavy pigmentation associated with polymerization, nor nitrile impurities resulting from isomerization). We report here the unexpectedly tight binding of these novel probes to both haemoglobin and myoglobin.

EXPERIMENTAL

Materials

Aliphatic and aromatic isonitriles were synthesized from the amine precursors (as obtained from Aldrich) by the phase-transfer-catalysed Hoffman carbylamine reaction (Gokel *et al.*, 1983). The following aromatic isonitriles were synthesized; phenylisonitrile (b.p. 55 °C/2 mmHg), methylphenylisonitrile (*ortho*, b.p. 90 °C/10 mmHg; *meta*, b.p. 46 °C/1 mmHg; *para*, b.p. 24 °C/0.3 mmHg), 2,6-dimethylphenylisonitrile (DIMPI; m.p. 72–73 °C), 2,6-diethylphenylisonitrile (DEPI; b.p. 70–73 °C/0.4 mmHg) and 2,6-di-isopropylphenylisonitrile (DIPI; b.p. 94–96 °C/0.7 mmHg). DIMPI was a white crystalline solid, the others were clear liquids at room temperature. All isonitriles were purified by vacuum distillation. Purities were estimated using g.l.c. (SE30 and OV225 columns), and were greater than 99%. Characteristic stretching frequencies of 2120 cm^{-1} were observed for the aromatic isonitriles.

Sheep haemoglobin, horse-heart myoglobin (type III) and Hepes were supplied by Sigma and were used as supplied. Sodium dithionite was obtained from Vine Chemicals, Widnes, Cheshire. Nitric oxide was made according to the method of Keilin & Hartree (1937). Carbon monoxide (purity 95%) and dimethylformamide (which was distilled prior to use to remove coloured impurities), were obtained from BDH, and oxygen (purity 95%) from BOC.

Ligand binding experiments

Protein solutions (approx. 50 μM according to haem) were made up each day in 0.1 M-Hepes buffer. The solutions were degassed under vacuum and then flushed with nitrogen.

Measurements were made in Sarstedt 4 ml gas-sealable plastic cuvettes sealed with Suba-Seals (size 29). Titration additions were made using a 1 μl Hamilton microsyringe.

Abbreviations used: DIMPI, 2,6-dimethylphenylisonitrile; DEPI, 2,6-diethylphenylisonitrile; DIPI, 2,6-di-isopropylphenylisonitrile.

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Spectrophotometric measurements were obtained using a Shimadzu UV/VIS 365 spectrophotometer.

A few grains of dithionite were added to cuvettes prior to addition of the protein solutions in order to scavenge any residual oxygen and to convert the ferric form of the protein to the deoxy ferrous form.

Titrations were performed by making sequential additions (typically 0.2–1.0 μl) of the isonitrile stock solution to the cuvettes. Titrations were followed by absorption peak and isosbestic point measurements after each addition. Changes in isosbestic points were used to correct base-line shift, but such adjustments were rarely necessary.

Attempts to displace ligands with carbon monoxide or nitric oxide were performed by titrating the protein to saturation with the isonitrile ligand and then adding a quantity of buffer saturated with the appropriate competitor. The reverse experiments were also performed by first saturating the protein with carbon monoxide or nitric oxide and then adding a saturating quantity of isonitrile.

Estimates of affinity were made using the relationship:

$$n = \frac{[CO]free \cdot K_a(RNC)}{[RNC]free \cdot K_a(CO)} \quad \text{where } n = \frac{[MbCO]}{[MbRNC]}$$

RESULTS

All seven phenylisonitrile probes were found to bind to both proteins giving the usual two-peak spectrum scanning from 450 nm to 650 nm. The wavelengths of the peaks for all ligands were 531 nm and 561 nm for haemoglobin and 532 nm and 567 nm for myoglobin. Fig. 1(a) shows the spectral change occurring during the course of a titration of myoglobin with DIMPI. In view of the instability of the monosubstituted ligands, further investigations were conducted using only the disubstituted ligands.

Fig. 1(b) shows a direct binding plot (i.e. fractional saturation versus total ligand concentration) comparing an aliphatic isonitrile (ethylisonitrile) with DIMPI. The affinity with which these aromatic ligands are bound is so high that normal binding plots were not possible: values of free ligand concentration could not be calculated. Phenylisonitrile and the monosubstituted ligands were not stable at room temperature and were excluded from any further investigation. The appearance of the binding plot for all ligands was not affected by temperature (between 20 and 40 °C) or pH (between 6 and 9).

Further investigations were conducted using only myoglobin as this avoided complications due to co-operative binding and the lability of the haemoglobin tetramer. Other methods of estimating the affinity of the aromatic isonitriles were sought. The affinities of oxygen, carbon monoxide and nitric oxide for horse heart myoglobin are 7.5×10^{-7} M, 3.4×10^{-6} M and 1×10^{-11} M (pH 7.0 at 25 °C; Mims *et al.*, 1983; Wittenburg and Wittenburg, 1981) respectively and were suggested as suitable competitors.

Displacement of DIMPI with oxygen, carbon monoxide and nitric oxide was slow and the results were somewhat variable. Ethyl isonitrile, which has the highest reported affinity of the aliphatic isonitriles (3.6×10^{-8} M; Mims *et al.*, 1983), underwent immediate 100 %$_n$ displacement by nitric oxide.

Attempts were made to obtain quantitative measurements using carefully monitored levels of competitor to displace isonitriles. Using this approach and measuring the proportion of each complex present at equilibrium yielded data which can then be used to get estimates of K_{RNC}.

This approach was attempted several times using either carbon monoxide or oxygen to displace both DIMPI and DEPI. The results showed a high degree of variation and were somewhat dependent on the competitor used. K values for DIMPI determined using oxygen, varied from 4 to 25 nM whereas carbon monoxide gave values ranging from 3 to 13 nM. For DEPI, only two determinations were attempted, giving values of 0.19 and 2.8 nM (using carbon monoxide). Attempts to estimate the K_{a_2} of DIPI by this method failed due to solubility problems which caused erratic baseline drift.

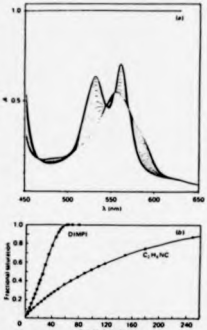

Fig. 1. (a) Repeated scan spectra made during the course of a titration of haemoglobin (50 μM haem in 0.1 M-Hepes, pH 7.0, 20 °C) with 5 μM increments of DIMPI and (b) Plot of fractional saturation versus total ligand concentration using data obtained from the titration shown in (a) and additional data from a titration of ethylisonitrile with haemoglobin

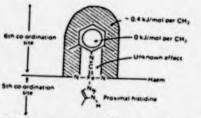

Fig. 2. Theoretical projection of DIMPI onto the schematic free-energy map of the haemoglobin binding site (Reisberg & Olson, 1980)

DISCUSSION

The tightest binding aliphatic isonitrile for myoglobin so far reported is ethylisonitrile (K_a, 3.6×10^{-4} M) and the weakest binding is *tert*-butyl isonitrile (K_a, 5.0×10^{-1} M) (Mims, 1983).

The high affinity of aromatic isonitriles observed in the present study is surprising especially when viewed in the light of the present model for human haemoglobin (Reisberg & Olson, 1980). Considering the size and positioning of the bulky alkyl substituents on the aromatic ligands it is reasonable to expect that considerable structural disruption must occur during binding to both haemoglobin and myoglobin. Ideally, the work described here should have been performed using the same protein as used by Reisberg & Olson (1980), however for reasons described earlier, our estimates of affinity were conducted using the simpler protein myoglobin. Strictly speaking, the Reisberg & Olson model is not fully applicable to our system. There is, however, sufficient structural homology between the two proteins to make some comparisons reasonable.

According to the model proposed by Reisberg & Olson (1980), the disubstituted aromatic isonitriles would be bound with their aliphatic side arms situated in the areas of high steric interaction (Fig. 2), suggesting that the ligand must meet with considerable steric hindrance at the binding site. What then is the driving force behind this unusually strong binding?

Reisberg & Olson conclude from their investigation that as ligand size is increased there is a roughly equal play-off between steric hindrance and hydrophobic attraction. But even if such a play-off were equal, the degree of affinity would, nevertheless, still be expected to be of the order of μM and not nM.

The overall free energy change resulting from ligand binding (ΔG) is taken to be (Reisberg & Olson 1980) the difference between the chemical potential of the bound ligand [*G*(Hbx)] and the chemical potential of the ligand in aqueous solution, *G*(x): $\Delta G = G(x) - G(Hbx)$.

Using the model of Reisberg & Olson (1980), the parameters ΔG, *G*(x) and *G*(Hbx) can be estimated. For example, the calculated value of *G*(x) for DIMPI is +1.27 kJ/mol. This can be calculated given that DIMPI has a relative surface area of approx. 1.82 (benzyl, 1.31; methyl, 0.51) using the Harris (1973) proportionality coefficient (0.97 kJ/mol per unit surface area) and subtracting 0.49 kJ/mol [which is the *G*(x) value of methyl isonitrile which has been arbitrarily assigned a value of zero].

A theoretical value of the bound potential, *G*(Hbx), of DIMPI in the model of Reisberg & Olson, is estimated by superimposing the structure of DIMPI on to the free-energy map of the haemoglobin binding site, and assuming that seven carbon atoms are likely to occupy the area of high steric interaction (0.4 kJ/C atom). Allowing for the *G*(Hbx) value of methylisonitrile (−1.12 kJ/mol) which represents the negative contribution from the formation of the metal–ligand bond, a value of +5.84 kJ/mol is obtained. This is an unreasonable large bound potential and would imply that, according to the model, DIMPI should not bind. Finally, given the relationship $\Delta G = G(Hbx) - G(x)$, the theoretical value for ΔG can be estimated to be +0.13 kJ/mol.

An observed value of ΔG and *G*(Hbx) can be obtained given the mean K_a for DIMPI (10×10^{-4} M) and the relationship $\Delta G = RT\ln K$. Hence, in our system, ΔG for DIMPI is −2.6 kJ/mol and *G*(Hbx) accordingly is −1.32 kJ/mol. Comparing the theoretical and observed ΔG values reveals a major discrepancy of 2.72 kJ/mol.

Similar calculations for DEPI (relative surface area 2.45; K 1 × 10^{-4} M) can be performed assuming that the terminal methyl groups of the alkyl side chains are pointing out of the area of highest steric interaction. Comparing theoretical and observed values of ΔG for DEPI reveals a discrepancy of 2.35 kJ/mol. This is

Table 1. Comparison of theoretical and observed free-energy parameters related to the binding of disubstituted aromatic isonitriles to myoglobin

R.S.A. is relative surface area (Reisberg & Olson, 1980).

	R.S.A.	Value (kJ/mol)			K (nM)
		G(x)	*G*(Hbx)	ΔG	
DIMPI					
Theoretical	1.82	+1.26	+1.40	+0.13	–
Observed	1.82	+1.26	−1.32	−2.58	10.0
DEPI					
Theoretical	2.54	+1.96	+1.40	−0.56	–
Observed	2.54	+1.96	−0.96	−2.92	1.0

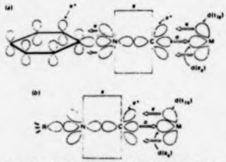

Fig. 3. Schematic representations of (*a*) metal–alkyl isonitrile bonding involving sigma and pi components and (*b*) metal–aromatic isonitrile bonding to show pi-delocalization between M[d(t_{2g})], NC(pi*), and aromatic ring pi*(e_{2u}) orbitals

comparable with the discrepancy seen for DIMPI (2.72 kJ/mol). Results for both ligands are shown in Table 1.

Accepting that the Reisberg & Olson model is extensive enough to account for the binding of large ligands, the *G*(Hbx) term is the most likely source from which the discrepancy in ΔG values may arise. The *G*(Hbx) term is calculated assuming that the isonitrile–iron bond strength is constant and is unaffected by the nature of the attached group. Whilst this assumption is reasonable when applied to aliphatic isonitriles, it may not be so for aromatic isonitriles, where there is substantial evidence for pi-interactions between the aromatic ring and the functional group; for the inorganic iron(II) aromatic isonitrile complexes $FeCl_2(DIMPI)_4$ and $[FeCl(DIMPI)_5]$-$FeCl_4$. X-ray structural data show mean iron–carbon bond distances of 1.895×10^{-1} nm and 1.906×10^{-1} nm respectively which are significantly shorter (5–10%) than those in corresponding iron(II) alkyl isonitrile systems. (Drew *et al.*, 1986).

When aliphatic isonitriles bind to metal ions they act both as sigma donors, (NC)sigma*$_{t_{1g}}$, metal $d(e_g)$, and as pi acceptors, metal $d_{pi}{}^{2}(t_{2g})$ (NC)(pi*); the latter relieves the build up of negative charge on the metal ion via empty accessible pi* orbitals on the ligand (Fig. 3*a*). These effects are synergic and lead to a strong metal–ligand linkage. Any sigma inductive effects between the aliphatic group (R) and the functional group (NC) are considered to be negligible and hence have little influence over the strength of the metal ligand binding. However in considering aromatic isonitrile binding to metal ions there is a significant difference, i.e. the proximity of the aromatic framework. In this case vacant ring orbitals of suitable energy and symmetry (pi*e_{2u}) can interact with the pi*(NC) orbitals of the isonitrile group and hence facilitate extensive delocalization of charge into the aromatic ring (Fig. 3*b*). Thus the ring becomes an effective electron sink, thereby increasing the pi-acceptor capacity of the ligand. Such an arrangement reinforces the strength of the metal–isonitrile bond.

It seems likely that this enhanced metal–ligand bond is responsible for the unexpectedly high affinity observed for aromatic isonitriles with both myoglobin and haemoglobin, and thereby accounts for the 10 kJ/mol discrepancy between predicted and observed G(Hbx) values.

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