Response of the frog olfactory system to controlled odour stimuli

T. MICHAEL POYNDER*

Presented at the 2nd Joint Perfumery Symposium organized by the British Society of Perfumers and the Society of Cosmetic Chemists of Great Britain at Eastbourne on 7–9th May 1973

Synopsis—The electrical events which occur in the nose of a frog when it is stimulated with ODORANTS have been studied.

For this study new techniques were developed for applying STIMULI of known composition and controlled concentration in a reproducible manner. The concentration and timing of the stimuli in the nose cavity has been monitored by means of a new device consisting of a sampling probe connected to a FLAME IONIZATION DETECTOR.

The ranges of concentration used have been wider than those reported previously and the form of the relationship between concentration and response size is now seen more clearly. It is that to be expected for a Langmuir type ADSORPTION of odorant molecules on the RECEPTOR surface.

INTRODUCTION

This investigation concerns the slow changes of electric potential which take place at the surface of an animal’s olfactory mucosa when it is stimulated by an odorant. These changes can be observed only in the region of the olfactory receptor cells and can be presumed to result from processes essential to olfactory perception. Their study should therefore help towards an understanding of the receptor mechanism.

In practice the changes in electric potential are recorded using a pair of electrodes, one of small tip diameter placed on the surface of the olfactory

*Bush Boake Allen Ltd, London E15 and the Department of Physiology, University College London, London WC1E 6BT.
mucosa, and the other larger one on inactive tissue. The plotted time course of the potential is called the ElectroOlfactogram or EOG. Fig. 1 illustrates a typical EOG resulting from a 5-s stimulus of 1,8 Cineole.

Figure 1. EOG resulting from a 5-s stimulus of 1,8 Cineole.

EOG's were first recorded by Hosoya and Yashida in 1937 (1). They were not systematically studied until nearly 20 years later. In 1956 Ottoson published his classic Analysis of the electrical activity of the olfactory epithelium (2). This was a lengthy and thorough investigation of EOG's in frogs. In it much attention was paid to the relationship of EOG size and shape to strength, duration and quality of the stimulus used. The conclusions of Ottoson's research remain practically unchanged today. This is a remarkable tribute considering the relatively simple apparatus which he had at his disposal.

It has been an object of the present research to look again and more closely at some of the factors investigated by Ottoson, taking advantage of modern instruments and technology to control and define as precisely as possible the chemical composition, concentration and time course of the stimuli used at the location where it matters—close to the olfactory epithelium.

Apparatus has now been evolved to meet these requirements (3) to a large degree. A feature of the stimulus applicator is that it can handle up to six different odour streams switching them on and off independently of each other and so close to the point of discharge that practically no time is
needed for a steady state to be reached. The apparatus may therefore be used for experiments which have not, it seems, been possible before. One of its first uses has been in the investigation of the relationship between stimulus concentration and the amplitude of the resulting EOG.

**METHODS**

*The animals and their preparation*

Common frogs (*Rana temporaria*) were used for the EOG recordings. A frog was first anaesthetized by placing it in 15 ml of 10% aqueous urethane in a beaker. As soon as the frog lost its reflexes, it was rinsed with water and placed in a holder. The frog remained anaesthetized by this treatment for the whole course of the experiment and it was not allowed to recover.

The olfactory epithelium was exposed by dissecting away the dorsal wall of the nasal cavity opposite to the eminentia olfactoria. The opening thus made was 2–3 mm across and provided access for the recording electrode and the stream of air carrying the stimulus.

*The stimulation system*

The stimulation system may best be described by reference to Fig. 2. It consists of a constant stream of clean moist air which plays on the olfactory epithelium all the time. This prevents the mucus from drying up, prevents

*Figure 2. Diagram of the arrangement of odorant streams.*
extraneous odours and, when required, carries the odour stimulus to the sensory area. This stream flows at 50 ml min$^{-1}$ and emerges from a jet 1 mm inside diameter in laminar flow. (Mean linear velocity = 100 cm s$^{-1}$ approx.)

The six odorant streams are switched into the carrier stream as close to the point of discharge as possible so that downstream there is minimum dead space and wall surface to delay delivery. The volume of the dead space is in fact about 0.15 ml so that with a flow rate of 50 ml min$^{-1}$ there would be a delay of 0.18 s before a stimulus reached 63% (= 1 - 1/e) of its full strength. This would represent the worst possible case, i.e. instantaneous forward mixing in the nozzle causing 'rounding' of the stimulus profile. (If there were no forward mixing in the nozzle, the stimulus would still take 0.18 s to reach the orifice but would arrive there at full strength.)

The odorant streams (up to six in number) are generated by passing clean dry air over pools of liquid odorants held in U tubes. These U tubes have a straight central portion so that the air stream passes over about 8 cm$^2$ liquid surface without bubbling through it. This prevents formation of spray which might be carried forward and upset the concentration. The air flow through each tube can be regulated from about 1 ml min$^{-1}$ to 5 ml min$^{-1}$ by controlling the pressure to a sintered stainless steel flow restrictor before the U tube—or up to 10 ml min$^{-1}$ by changing the flow restrictor. At these small flow rates the vapour leaving the U tube is practically in equilibrium with the liquid odorant. If necessary the U tubes can be immersed in a water bath to keep their temperature constant at or below room temperature.

The odorants used are chemicals whose purity has been checked by glc analysis of head-space samples. They are used either neat or diluted with water or paraffin oil. (The paraffin oil used is first deodorized by treatment with activated silica.) This dilution is the means most used to provide widely different rates of delivery of odorant. The rates can also be regulated by adjusting the air flow rates or by cooling the U tubes in order to lower the vapour pressures.

The odorants are conveyed to the applicator (where they are switched into the main carrier stream) by means of PTFE tubing of inside diameter 0.4 mm. This tubing is conveniently flexible and is easily and cheaply replaceable. It does absorb some of the odorant but, some minutes after starting the flow, it reaches a steady state which is not disturbed by the switching operation since the flow is not thereby interrupted. It is an important feature of the design of the system that this should be so. All
changes in flows and concentrations are confined to the switch and nozzle of the applicator itself. Contamination of one odorant by another and adsorption effects are therefore reduced to a minimum.

The function of the applicator is to enable odorant streams to be added to and mixed with the main carrier stream so that odor stimuli of predetermined duration and sequence can be directed into the frog's nasal cavity. The applicator, which is illustrated in Fig. 3, consists of three parts. These are the stream switching part, the mechanism for operating the switches and the nozzle which mixes and directs the gas stream towards the animal. The switches and nozzle are shown on a larger scale in Fig. 4. There

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**Figure 3.** Scale drawing of six-channel odour applicator. (Saggital section, channel 1 off, channel 4 on.)

**Figure 4.** Detail of applicator stream switching system, showing channel 1 'off' and channel 4 'on'. (Glass nozzle on right is not all shown.)
are six switches, one for each odorant stream. Each switch operates on the following principle. The carrier gas divides at a T junction one branch of which leads to a vent while the other leads to the nozzle and the frog. Odorant is introduced into one or other branch of the T by means of a movable, loose-fitting inner tube. It is swept by the carrier either to vent or to the nozzle according to which branch the orifice of the odorant tube is moved into.

The switches are designed so that each can be actuated independently of the others. Carrier gas flowing at $100 \text{ ml min}^{-1}$ splits into six radially disposed ducts. Each of these then divides at one of the abovementioned T junctions. Therefore $100/12 \text{ ml min}^{-1}$ gas issues from each of the 12 branches of the T's. Six of these streams recombine as they enter the nozzle which therefore delivers $50 \text{ ml min}^{-1}$, while the other six lead away to a vent. Odorant streams not being applied and escaping at the vent are prevented from entering the room by an extraction duct.

The movable, loose-fitting inner tubes which convey odorant into branches of the T's are stainless steel tubes of outside diameter 0.3 mm. They are moved parallel to their axes by means of pistons which are fixed to them and which run in cylinders. The cylinders are each connected at one end to a source of compressed air or to suction. The change from compressed air to suction is made by solenoid valves which are actuated according to a preset programme by a timing device.

The nozzle of the applicator is designed to ensure good mixing of the odorant into the carrier stream. At first various designs were tried in which baffles were inserted in the nozzle to break up the flow. Surprisingly these did not bring about the desired mixing. The best way was found to be to rely on diffusion and the problem was simply and effectively solved by extending the 1 mm bore outlet tube from 1 cm to 3.5 cm in length. The fact that the gases were not mixed properly in the original 1 cm long outlet tube was revealed by means of the vapour monitoring system described below.

Monitoring system for the odour stimuli (4)

This part of the apparatus is shown diagrammatically in Fig. 5. It is used to indicate the concentration of organic vapours in any particular locality such as in the stream issuing from the applicator jet. It consists essentially of a short probe tube connected directly into a standard flame ionization detector (fid) supplied by Pye Unicam Ltd. A sample of the vapours to be tested is drawn continuously into the fid through the probe by applying suction to the fid outlet. The probe tube is 4 cm long and 0.4 mm outside
diameter (od). The flow of sample being withdrawn by this probe needs to be small compared with the whole flow of gases being sampled. On the other hand the sample flow needs to be large enough for low concentrations of vapour to be measured by the fid. A sample flow rate around 2 ml min$^{-1}$ is normally used. The transfer of sample towards the fid is slowed up by adsorption of organics on the inside walls of the probe. In order to minimize this delaying effect, the hydrogen for the flame is also introduced via the probe tube. This is done by means of a smaller tube (0.3 mm od) the end of which is bent and hooked $\frac{1}{2}$ mm into the open end of the probe. The probe inlet is thus nearly closed and offers resistance to sample flow such that a suction of about 50 cm water gauge needs to be applied to the fid exhaust to maintain this flow.

The suction (50 cm water gauge) needs to be adjustable and free of drift and pulsations. This is achieved, as shown in Fig. 5, by means of an adjustable dip-leg submerged in water and a 3 litre bottle to smooth out pressure pulses. The exhaust from the fid leads downhill to a trap to catch water formed in the flame. The fid itself is lagged to prevent condensation of water vapour inside it, to keep the fid at a steady temperature and to stop heat radiation affecting the frog.

Figure 5. Diagram of monitoring system for odour stimuli.
The vapour monitoring system has been used mainly for determining the concentration of odour stimuli delivered by the applicator. Its fast response facilitates the adjustment of the odorant streams so that the desired concentrations are delivered. Once the odorant solutions and flow rates have been adjusted, it is unnecessary always to check the concentrations during a series of olfactory response measurements but only before and after the series. Only when solutions of very volatile substances are used is there any change noticeable in the composition over the period of an experiment.

The vapour monitoring system has other important uses. One of these, as mentioned above, was to check the mixing of odorant and carrier streams in the applicator jet. This was done by moving the probe tip 0.2 mm at a time across the 1-mm diameter of the jet orifice with an odorant switched on. The concentration profile so obtained was asymmetrical when mixing was not complete. Incidentally this experiment demonstrated that the sampling system was capable of discerning differences between points only 0.2 mm apart.

When the concentration profiles are determined for diameters progressively further and further away from the jet, a three-dimensional picture may be built up showing concentration on the vertical scale and displacement from the jet axis on the horizontal scale. Such a diagram is shown in Fig. 6. This may be the first time that an odorant jet stream has been mapped in this way and it is interesting to note that the concentration has fallen to half its original at a distance 12 mm from the orifice due to the spreading sideways of the odorant. Obviously the positioning of the applicator jet relative to the nasal cavity is of considerable importance. It would be useful to know also how the humidity of the jet stream—originally saturated—falls off for the same reasons. The olfactory mucus does sometimes become dry in spite of the precaution of saturating the carrier gas with water vapour.

Another use of the vapour monitoring system has been to check the efficiency of the applicator switching and to obtain some idea of the time course of build-up and fall-off of concentration when an odorant is switched on and off. It was found that, even with much smaller carrier gas flow and increased odorant stream flows, the device reliably and completely switched on and off. Fig. 7 shows the three fid response curves to 2-s applications of acetone, cineole (strong) and cineole 1 000 times more dilute. Ideally the traces should be rectangular. The rounding of the shoulders is due largely to the slow movement of sample up the probe tube
Figure 6. Concentration profile of applicator jet stream shown in 3-dimensional perspective. Measured by Fido.

Figure 7. Response of the fid monitoring system to 'square' pulses of odorants lasting 2 s. Horizontal axis is time, vertical concentration.
Elution curves can be used for measuring flow rates with minimal back pressure.

Figure 8. Arrangement showing how the vapour monitoring system can be used for measuring gas flow rates.

due to adsorption on the walls. That this was so was shown by applying a 'square' odorant pulse by a different method in which there was no possibility of a 'rounded' application. The response curves were only slightly sharper than before. It is interesting to observe, in Fig. 7 the marked increase in adsorption effects when dealing with the more dilute odorant. This emphasizes the need for an applicator system such as that described here especially when studying physiological responses to very dilute odour stimuli.

The concentration time courses of odorants delivered by the applicator have more recently been studied by directing the jet stream directly into an improvised fid having a cut-away side for access. The time constant of the applicator has been shown to be about 0.12 s or more depending on the material being handled.

Yet another use of the vapour monitoring system has been in the measurement of gas flow rates without appreciably increasing the resistance to their flow. This has been done by using the flow to be measured to elute
an organic vapour from a nearly enclosed space of known volume. The concentration in this enclosed space is kept uniform by mechanical mixing (if diffusion alone is not adequate) and is measured continuously by the vapour monitor. The arrangement is illustrated in Fig. 8. The volume of enclosed space divided by the time constant of the exponential fall in concentration gives the flow rate of the eluting gas. This method has been used with advantage to measure the flow of gas from the applicator nozzle where back-pressure of most conventional flow meters would have altered the flow being measured.

This concludes the description of the apparatus for generating, applying and monitoring odour stimuli. It has been somewhat detailed because it is hoped that some of the ideas will be useful to those researching in this field and perhaps to others also. Moreover it seems desirable to place more emphasis on this side of any study of stimulus–response relationships than has been accorded hitherto. There is now the means of investigating more thoroughly firstly the relationship between stimulus concentration and physiological response, secondly the interactions between different odours applied simultaneously or sequentially and thirdly the time course of physiological events following an odour stimulus. All these can provide useful information about olfactory receptor mechanisms and it is with the first that the experimental part of this paper is concerned.

The electrode system for recording EOG’s

The electrodes used for recording EOG’s were similar to those used by Ottoson (3). The recording electrode was a Pyrex glass pipette having a tip diameter of 50–100 μm and filled with normal saline containing 2% agar. Electrical connection was through a AgCl–Ag wire inserted in the pipette. The second electrode was a AgCl–Ag plate wrapped in lint, soaked in normal saline and inserted in the frog’s mouth.

The signals picked up by these electrodes were led to a high input resistance preamplifier, a main amplifier and the EOG’s recorded on a strip chart.

Procedure for EOG experiments

In each experiment one frog was used for the study of EOG’s resulting from one odorant chemical at different concentrations. The vaporizer tubes were charged with the odorant made up to different dilutions with a suitable solvent (usually deodorized light paraffin oil). Typically the dilutions were chosen so that the applicator delivered a set of stimuli in which each was 10
times weaker than the preceding one. Stimuli of intermediate strength were obtained by altering the gas flows in each channel by a factor of three. In this way stimuli having concentrations covering up to four decades in half decade steps were available.

The actual concentrations of stimuli were, of course, measured by the vapour monitoring system and this was done just before an experiment with a frog. Arbitrary units were used since for the purpose of the present investigation this was all that was necessary. In the case of certain odorants the lowest concentration gave fid signals obscured by noise and several readings had to be averaged to arrive at an estimate.

An anaesthetized frog was prepared and placed in a head holder. The applicator was positioned with its nozzle pointing into the opened nasal cavity and about 4 mm away from it. The recording electrode was lowered by means of a micromanipulator so that its tip just touched the surface of the mucus overlying the eminentia olfactoria.

The recordings required for this investigation were of the peak EOG voltages which are in fact reached soon after the onset of stimuli (see Fig. 1). Stimuli could therefore be switched off as soon as this peak voltage had been reached and doing this helped to minimize fatigue effects especially for strong stimuli. Also to combat fatigue 2 or 3 min were allowed for recovery between each stimulation with the higher concentrations.

The stimuli were usually applied in both ascending and descending order of concentration so that two EOG readings were obtained at each concentration.

The odorant chemicals for which EOG data are reported in this paper are:

Amyl acetate,
1,8 Cineole,
Linalol, and
Butyl acetate.

RESULTS

The results are shown entirely in the form of graphs on which all the experimentally determined points are plotted. These graphs are shown in Figs. 9-13.

The abscissae in every case are the logarithms (base 10) of the concentrations in arbitrary units of the stimuli used. The EOG amplitudes in millivolts are plotted as the corresponding ordinates in Figs 9(a), 10(a), 11(a),


Figure 9. (a) EOG (mV) v. log concn. Amyl acetate. EOG max = 7.9 mV. (b) log EOG v. log concn. Amyl acetate.
Figure 10. (a) EOG (mV) v. log concn. 1, 8 Cineole. EOG max = 6.8 mV. Conc. for half max EOG = 3.5. (b) log EOG v. log concn. 1, 8 Cineole.
Figure 11. (a) EOG (mV) v. log concn. 1, 8 Cineole. Repeat experiment. EOG max = 6.0 mV. Conc. for half max EOG = 3.5. (b) log EOG v. log concn. 1, 8 Cineole.
Figure 12. (a) EOG (mV) v. log concn. Linalol. EOG max = 8.4 mV. (b) log EOG v. log concn. Linalol.
Figure 13. (a) EOG (mV) v. log concn. Butyl acetate. EOG max = 15.8 mV.
(b) log EOG v. log concn. Butyl acetate.
Figures 9(b), 10(b), 11(b), 12(b) and 13(b). Thus for each experiment there is a pair of graphs representing the same data in two different ways:
(a) log stimulus v. response, and (b) log stimulus v. log response.

The curves drawn on the graphs represent a postulated mathematical relationship between stimulus and response analogous to the Langmuir adsorption isotherm (5). (See Discussion.) This postulated relationship contains only two arbitrary constants which define (a) the EOG for infinite stimulus concentration, and (b) the stimulus concentration required for half this (hypothetical) maximum EOG. These constants have been chosen in the case of each experiment to make the curve fit the experimental points as closely as possible. The values of the maximum EOG’s are shown in the legends under each figure.

**DISCUSSION**

In order to discuss the significance of the foregoing results it will be helpful to have a picture in one’s mind of the events as far as they are known which give rise to an EOG.

The origin of the potential is the electrical polarization which exists between the inside and outside of cells forming the olfactory epithelial layer. This layer is formed by a mosaic of mainly two kinds of columnar cells. One kind is the receptor cells and the other the supporting cells. Odorant molecules in the mucus overlying these cells spread by diffusion and interact with receptor sites which form some part of a cell membrane. This interaction results in an increase of membrane permeability to certain ions. An ionic current then flows which depolarizes the receptor cell. (It is this depolarization of the receptor cell which causes it to generate action potentials.) The ionic current flows in a circuit through receptor cell, supporting cell and overlying mucus. Each of these components of the circuit contributes some resistance so that there is a potential difference between the surface of the mucus and the base of the receptor cells. The EOG is the summed effect of the 5 000 or so receptor cells which lie within range of the electrode tip. The magnitude of the EOG can therefore be considered in terms of the local circuits at cellular level.

Suppose that the EMF of the circuit is $E$, that the active membrane has a variable resistance, $R_m$, depending on presence of odorant and that the other resistances of mucus and tissue total $R_t$ which is constant.
Then the potential, $V_t$, across the constant resistance, $R_t$, is given by

$$V_t = E \frac{R_t}{R_t + R_m}.$$  

If membrane conductance, $G_m$, is $1/R_m$ then

$$V_t = E \frac{R_t G_m}{1 + G_m R_t}.$$  

If it is assumed that the membrane conductance is a linear function of odorant concentration, $G_m = k.c$, then

$$V_t = E.R_t \frac{kc}{1 + kc R_t}.$$  

$V$ is to be our estimate of the EOG so that

$$EOG = \frac{Ac}{1 + Bc}$$

where $A$ and $B$ are constants.

Up to this point the argument has followed that of Tucker and Shibuya (6). But the above assumption that the membrane conductance is a linear function of concentration is clearly not correct because a saturation must soon be reached. Instead let us assume that there is a limited number of gates in the membrane corresponding to a maximum conductance of $G_M$.

Then, analogous to the Langmuir adsorption isotherm, we have

$$G_m = G_M \frac{kc}{1 + k}$$

where $k$ is a constant.

Combining this with equation (1) we get

$$V_t = \frac{E.R_t G_M.k.c}{1 + (k + G_M.R_t.k)c}$$

so $EOG = \frac{Ac}{1 + Bc}$ where $A$ and $B$ are constants as before.  

Therefore it may after all be expected that the EOG's would follow equation (2).

The curves plotted on the graphs of results are in fact those obtained from equation (2) with values of $A$ and $B$ chosen to make them fit as well as
possible the experimental points. The curves fit well especially having regard to the wide range of concentrations used and there is no evidence to suggest that the relationship is other than that deduced.

Earlier researchers (2, 7 and 8) have worked with smaller concentration ranges. When their results were plotted on log–log axes, the points fell approximately on straight lines implying a power function relationship. The lines for different substances had different gradients and it was thought that the gradients (exponents) were characteristic of the substance.

The results of the present study have also been plotted on log–log axes and it can be seen that for low concentrations the relationship is indeed practically linear. However, in no case is there suggestion that the gradient is other than 1 for low concentrations. Another feature of the results is that there is no evidence for a threshold concentration below which there is no EOG response.

In conclusion it may be noted that the sense of smell now seems to fall into line with other senses in following a stimulus–response relationship which is sigmoidal in form when plotted on semi-log paper.

ACKNOWLEDGMENTS

I would like to thank my employers, Bush Boake Allen Ltd, for allowing me to pursue this research and for grants for equipment. I also thank the Medical Research Council for grants for equipment and colleagues at University College London for help and encouragement—especially Hugh Bostock.

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