A Chemical-Modification Approach to the Olfactory Code

STUDIES WITH A THIOL-SPECIFIC REAGENT

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(Received 17 July 1978)

The effects of thiol-specific reagents on the amplitude of the electro-olfactogram (E.O.G.) responses elicited from frog olfactory mucosa by pulses of odorant vapours was studied. The impermeant thiol-specific reagent mersalyl [(3-{[2-(carboxymethoxy)-benzoyl]amino}-2-methoxypropyl)hydroxymercury monosodium salt] brings about a rapid decrease in the E.O.G. signal obtained with the odorant pentyl acetate. The extent of the decrease is proportional to the concentration of the mersalyl applied and the effect of the reagent is partially but incompletely reversed by treatment of the labelled mucosa with dithiothreitol. The sites labelled by mersalyl can be protected by pretreating the mucosa with a dilute solution of the odorant pentyl acetate and leaving the solution in contact with the tissue after the addition of mersalyl. When the protecting odorant is washed out of the tissue, the original E.O.G. amplitude is regained. Pentyl acetate applied to the mucosa protected the E.O.G. response to vapour pulses of the following odorants from the effects of mersalyl: *n*-butyric acid, *n*-butyl acetate, phenylacetaldehyde and cineole (1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane). The pentyl acetate applied to the mucosa failed to protect the E.O.G. response to vapour pulses of the following odorants from the effects of mersalyl: butan-1-ol, benzyl acetate, nitrobenzene, β -ionone and linalyl acetate. The significance of the differential protection effects for the odour-quality-coding mechanism in the olfactory primary neurons is discussed. It is suggested that the olfactory code at this level of the olfactory system may be elucidated by chemical-modification methods.

There are two outstanding problems concerned with molecular mechanisms in olfaction. First, there is the nature of the transduction mechanism, which converts the initial odorant-membrane interaction into a receptor potential in the primary neurons. Secondly, there is the nature of the coding mechanism in the primary neurons, which allows the olfactory system to recognize and discriminate between different odours. There have been many speculations on possible mechanisms for both these steps in olfaction (Beidler, 1971; Poynder, 1974), but there is little experimental evidence to support the proposed mechanisms. We have put forward a specific mechanism, the allosteric-membrane enzyme hypothesis, which can explain both the transduction and the coding in the membranes of the olfactory primary neurons (Dodd *et al.*, 1978). In this hypothesis odorants are portrayed as allosteric regulators of a cyclic nucleotide-dependent enzyme

system in the plasma membranes of the olfactory cilia. This mechanism is supported by several types of experimental evidence, and in the present paper we offer further evidence from chemical-modification studies on the discrimination mechanism in the sensory membranes.

The discrimination mechanism in the olfactory system appears to operate at several distinct levels (Beidler, 1971). In the present studies we are interested in the recognition and coding steps that take place in the plasma membranes of the primary neurons. The primary neurons of vertebrates behave as generalist cells and they respond to a wide variety of odorants. Electrophysiological evidence indicates that the response patterns of no two cells to a range of odorants are identical, indicating a weak discrimination at the level of the first neurons in the system. The secondary neurons in the olfactory bulb, which synapse with several thousand primary neurons, show a much more specific response to odorants. The behaviour of the primary neurons has been explained on the basis that there are a number of olfactory receptor proteins, which are distributed in

Abbreviations used: E.O.G., electro-olfactogram; mersalyl, (3-{[2-(carboxymethoxy)benzoyl]amino}-2-methoxypropyl)hydroxymercury monosodium salt.

different ways on the primary neurons (Gesteland, 1976).

We have suggested that the olfactory receptor proteins are probably regulatory subunits of a membrane enzyme system in the plasma membrane of the primary neurons. Odorants would then be regarded as regulatory ligands, and we could invoke a variety of regulatory mechanisms (Dodd, 1976; Dodd *et al.*, 1978) to explain the coding at the level of the sensory membrane. Further, the experimental methods that have been used for studying regulatory enzyme systems (Koshland, 1970), including chemical-modification methods, could then be expected to elucidate the olfactory code. The code can be approached by identifying the number of independent types of olfactory-receptor proteins, and then by studying the possible types of interaction between these proteins that can take place in the membrane.

Chemical-modification methods appear to be an attractive approach to the olfactory code, since they have been successfully used with a variety of systems including enzymes (Shaw, 1970; Brocklehurst *et al.*, 1970), membranes (Barratt *et al.*, 1969; Tinberg & Packer, 1976), bacterial chemoreceptors (Barber, 1976), protozoan chemoreceptors (Doughty & Dodd, 1978), invertebrate olfactory receptors (Villet, 1974; Frazier & Heitz, 1975) and vertebrate olfactory receptors (Getchell & Gesteland, 1972; Menevse *et al.*, 1977a). In studies on frog olfactory receptors with *N*-ethylmaleimide, Getchell & Gesteland (1972) found that they could label specific odorant-binding sites and that the labelling could be prevented by the odorants that were specifically bound to these sites. Two points in these experiments require further clarification. First, *N*-ethylmaleimide can permeate rapidly through membranes and the results obtained in these experiments might be due to labelling of an intracellular protein. Secondly, very high concentrations (12.5 mM) of the protecting odorants were used and under these conditions a variety of changes in the olfactory membranes might have occurred. In the experiments reported here, we have carried out specific chemical modification on intact frog olfactory mucosa with a non-penetrant thiol-specific reagent, mersalyl, and with lower concentrations of competing odorants. The results show that we can label specific odorant-binding sites and that the labelling of these sites could be prevented by some types of chemicals, but not by others.

Materials and Methods

Chemicals

N-Ethylmaleimide was purchased from Eastman Kodak Co., Rochester, NY, U.S.A. Mersalyl (sodium salt), dithiothreitol and Triton X-100 were purchased from Sigma Chemical Co., London, U.K. Pentyl

acetate, cineole, *m*-xylene and phenylacetaldehyde were purchased from BDH Chemicals, Poole, Dorset, U.K.; ethyl *n*-butyrate, naphthalene and *n*-butyric acid were obtained from Aldrich Chemical Co. Gillingham, Kent, U.K.; *n*-butyl acetate, butan-1-ol, nitrobenzene, β -ionone and benzaldehyde were purchased from Fison's Chemical Co., Loughborough, Leics., U.K. Linalyl acetate was a gift from Hoffman-LaRoche, Basle, Switzerland. The inorganic salts and buffer materials were purchased from BDH Chemicals. The purity of the odorants as estimated by g.l.c. analysis on 20% DEGS Chromosorb W/HP in a Perkin-Elmer F11 chromatograph was in excess of 98%.

Animals

The common frog (*Rana temporaria*), obtained from Gerrard Biological Supply (East Preson, West Sussex, U.K.), was used. They were kept for a period of days in a cold-room (4°C) after arrival, and each frog was warmed at room temperature (19–25°C) for at least 2 h before each experiment. Both sexes were used.

General methods

All solutions were made up in double-glass-distilled water. Amphibian Ringer's solution was used for experiments involving protein reagents and drugs. This Ringer's solution had the following composition: 111 mM-NaCl; 1.9 mM-KCl; 1.1 mM-CaCl₂; 24 mM-NaHCO₃. All solutions used were pH 7.0 unless otherwise stated. A solution of 0.5% methanol in Ringer's solution was used as the control buffer for the experiments in which a solution of pentyl acetate was applied to the olfactory mucosa. The control buffer for experiments with this reagent was a 1:1 (v/v) solution of the buffer and Ringer's solution: the pH was adjusted to 8.0. Solutions of enzymes and reagents were always freshly prepared on the day of use.

Olfactometer

We constructed an olfactometer from the design of Poynder (1974). In this instrument a stream of clean moist air (50 ml/min) plays on the olfactory epithelium all the time. The six odorant streams are switched into the carrier stream under conditions that give a square-wave stimulus pulse of vapour. The odorant streams are generated by passing clean dry air over pools of liquid odorants held in U-tubes. Details of the instrumentation are given elsewhere (Bostock & Poynder, 1972; Poynder, 1974; Menevse, 1977).

Electrophysiological methods

The frogs were either pithed or anaesthetized; both methods of preparation gave similar results. The

animals were anaesthetized by immersion in 15 ml of urethane solution (10%, w/v, in water), absorption being arrested by rinsing the skin with tap water as soon as the reflex responses to eye poking and skin tweaking were abolished. The frogs remained anaesthetized by this treatment for the whole course of the experiment and they were not allowed to recover.

The olfactory eminence on one side was exposed by cutting away the skin, cartilage and finally the epithelium of the roof of the nasal cavity, great care being taken to prevent any spillage of blood on to the eminence. The frog was covered with a moist cloth and fixed by ear bars in a Perspex frame, which was placed in position beneath the adjustable odour applicator and electrode holders. A frog with good blood circulation was essential if stable electro-olfactogram recordings were required over several hours.

For experiments in which solutions of reagents and pentyl acetate were applied to the olfactory eminence, a Teflon strip was used to block the buccal aperture of the internal naris. This prevented drainage of solutions from the olfactory cavity.

The reference or ground electrode was an AgCl/Ag wire coiled at one end, wrapped in muslin, soaked in 0.9% NaCl and inserted in the animal's mouth. The exposed length of silver wire was sealed in Teflon tubing. The recording electrode was a micropipette, pulled from Pyrex tubing (1 mm internal diam.), to produce a smooth tip of diameter 30–100 μ m. It was filled with either 0.9% NaCl or 3 M-KCl containing 2% agar. For the experiments in which protein reagents were used, agar was omitted and the electrode was filled only with 0.9% NaCl or 3 M-KCl. Electrical connection was through an AgCl/Ag wire inserted in the pipette. Each electrode had a resistance of about 0.3–1.0 M Ω , and lasted for three or four experiments. All pipettes were examined under a low-power microscope before each experiment to ensure that the filling process had been carried out correctly. The signal from the electrodes was led to a high-impedance amplifier and was displayed on a chart recorder.

The applicator of the olfactometer was positioned with its nozzle pointing into the opened olfactory cavity and about 5 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 olfactory eminence. Three test puffs of the stimulus were delivered and their E.O.G. responses were recorded to determine whether the receptors were responding normally. The odour-dilution factor for the odorants was determined to find a vapour-phase odorant concentration that would give a half-value of the maximum E.O.G. amplitude found with a particular odorant. All stimulations were between 5 and 10 s duration and an interval of

2–3 min was allowed for recovery between each stimulation.

For the liquid-phase experiments in which group-specific protein reagents or pentyl acetate were applied to the olfactory eminence, the following procedure was carried out. After delivery of the test puffs, the recording electrode was lifted by means of a micromanipulator, and its tip placed in a beaker of Ringer's solution. The olfactory cavity was filled with a buffer solution or Ringer's solution, and the olfactory eminence was always completely covered with solution. Disposable syringes (1 ml) with stainless-steel needles were used to fill and drain the olfactory chamber. Separate syringes and needles for filling and draining were used and they were very gently introduced in to the cavity. The needles were placed to one side of the olfactory eminence to avoid mechanical damage to the tissue. The solution was left in the cavity for a specified time (usually 10 min) and then changed. Finally, after withdrawal of the solution, the recording electrode was lowered to the same spot as for the test puffs, and three E.O.G. responses were elicited. These responses were used as the control responses. The micropipette was again lifted and its tip was placed in Ringer's solution. The olfactory cavity was then filled with the solution of the protein reagent; this solution was changed in the same way as the control solution. After a specified time, this solution was withdrawn, and three E.O.G. responses were elicited to determine the effects of the reagent on this response.

To test the degree of reversibility of the effects of the reagent, the electrode was lifted and the cavity was again filled with the Ringer's solution for a given time, as described above. This procedure is referred to below as washing the mucosa. The cavity was then drained, the electrode was lowered and three E.O.G. responses were elicited.

Protocol for protection experiments

The protection experiments were carried out by following the basic design of Getchell & Gesteland (1972). 1. An E.O.G. response to pentyl acetate was recorded from the untreated mucosa to establish that the frog was responding normally. 2. The nasal cavity was filled with Ringer's solution containing 0.5% methanol; the solution was withdrawn and replaced after 2, 5 and 8 min. After 10 min the solution was withdrawn. 3. Three E.O.G. responses to pentyl acetate were recorded. The average of the amplitudes to these three E.O.G. responses was the control amplitude (100%). 4. The mucosa was exposed to one of the following sequences of treatments. (a) Ringer's solution containing 0.5% methanol for 12 min. The solution was changed after 4 min and 8 min, and after 12 min the solution was withdrawn. (b) Pentyl acetate (1 mM) in Ringer's solution containing 0.5%

methanol for 12 min; the solution was changed after 4 min and 8 min, and after 12 min the solution was withdrawn. (c) Ringer's solution + 0.5% methanol for 4 min; mersalyl (0.1 mM) for 4 min; Ringer's solution + 0.5% methanol for 4 min. (d) Pentyl acetate (1 mM) in Ringer's solution + 0.5% methanol for 4 min. Pentyl acetate (1 mM) + mersalyl (0.1 mM) in Ringer's solution + 0.5% methanol for 4 min; pentyl acetate (1 mM) in Ringer's solution + 0.5% methanol for 4 min. 5. The nasal cavity was filled with Ringer's solution + 0.5% methanol as in step 2. 6. E.O.G. responses to pentyl acetate were elicited and recorded every 3 min for the next 39 min.

Results

Effect of mersalyl and other thiol-specific reagents on the E.O.G. response

The first characteristic electrical response of a sensory neuron after stimulation is the production of a receptor potential and, in general, this is the most appropriate plasma-membrane parameter that should be monitored in chemical-modification experiments on the sensory membrane. In the case of vertebrate olfactory receptors it is very difficult for technical reasons to record the receptor potential from a single neuron by using a microelectrode. However, by using a larger extracellular electrode it is possible to measure the summated receptor potentials from some hundreds of neurons. The response obtained in this way is called the electro-olfactogram (E.O.G.) and it has the essential features of a receptor potential (Ottoson, 1956). With our design of olfactometer, it is possible to use a square-wave puff of odorant as a stimulus. The response to such a stimulus is a characteristically shaped E.O.G. (Fig. 1) with distinct peak and plateau regions. This shape resembles the receptor potentials of the sensory cells of other senses in response to comparable stimuli (Poynder, 1974). The peak/plateau ratio is a characteristic feature of the E.O.G. for a particular odorant at a particular concentration and this ratio possibly reflects the transduction mechanism in the plasma membrane.

For the experiments reported in the present paper, we have concentrated on pentyl acetate as a suitable odour stimulus. Most frogs respond well to this odorant and give E.O.G. responses of large magnitude with well-defined peak and plateau regions (Fig. 1). It is chemically unreactive under the conditions used in these experiments, and its high vapour pressure ensures that it is an easy odorant both to apply to the mucosa and also to remove from the mucosa. We chose to use thiol-specific reagents to label an olfactory receptor protein. The rationale for this decision is that the plasma membrane of the olfactory neurons probably contains phosphatidylethanolamine, which has a reactive amino head-group, and also glycoproteins,

Fig. 1. Time course of the effects of 0.1 mM-mersalyl in Ringer's solution, pH 7.0, on the E.O.G. responses to pentylacetate

The square-wave inset shows the concentration-time profile of the stimulus vapour pulse.

Fig. 2. Time course of the development of the effects of mersalyl in Ringer's solution, pH 7.0, on the E.O.G. amplitude to pentylacetate

The results are expressed as the means $\pm$ S.E.M. for the numbers of animals used in parentheses. Symbols: ■, Ringer's solution + 0.5% methanol (1); ●, 1 μ M-mersalyl (2); ○, 0.1 mM-mersalyl (4); △, 1 mM-mersalyl (3); □, 10 mM-mersalyl (2).

which contain hydroxy groups, and thus a reagent directed against either of these groups is less likely to specifically label an olfactory-receptor protein. Extensive chemical modification experiments with a variety of group-specific reagents as well as hydrolytic enzymes (Menevse, 1977) show that the sensory membrane can be modified by labelling or altering several types of functional group. The thiol-specific reagents *p*-chloromercuribenzoic acid, *N*-ethylmaleimide and mersalyl, when applied in solution to the intact olfactory mucosa in the olfactory cavity, brought about an irreversible decrease in the E.O.G.

response to the odorants pentyl acetate and cineole. The results obtained with *N*-ethylmaleimide were similar to those obtained by Getchell & Gesteland (1972) with a related odorant ethyl *n*-butyrate. Of the three reagents used, we chose mersalyl for more detailed studies as it is a non-penetrant chemical (Mavier & Hanoune, 1975).

Chemical modification of the sensory membranes can be carried out by using vapour-phase pulses of *N*-ethylmaleimide and other volatile group-specific reagents (Menevse *et al.*, 1977a; Menevse, 1977). Mersalyl is insufficiently volatile to be used in the vapour phase.

A solution of mersalyl brought about a rapid decrease in the amplitude of both the peak and plateau phases of the E.O.G. response to pentyl acetate (Figs. 1 and 2). The application of Ringer's solution alone did not change the E.O.G. (Fig. 2), and showed that the gentle application of solutions to the mucosa, with the methods we have chosen, neither damages the tissues nor alters the response to a normal vapour-phase odorant stimulus. Since the biochemical steps underlying the different phases of the E.O.G. response are not known, we have characterized the response of the olfactory neurons empirically by reporting the relative amplitude of the E.O.G. response peak in subsequent experiments.

The extent of inhibition of the E.O.G. response increases with increasing concentration of mersalyl (Fig. 2), but it is not abolished at the highest concentration used. At each concentration there is an initial rapid decline in the E.O.G. that leads to a final degree of inhibition that is characteristic for each concentration of mersalyl. The decline in the E.O.G. could not be reversed by washing the mucosa with Ringer's solution for 20 min (Fig. 3), but was partially reversed by washing with dithiothreitol (Fig. 3). The actual extent of reversal may be greater since dithiothreitol alone at the concentration used decreased the amplitude of E.O.G. to $70 \pm 9\%$ (mean $\pm$ S.E.M. for four frogs) of the original amplitude.

The characteristic stable plateau reached at each concentration of mersalyl indicates that, despite the high concentrations of the reagent used, a distinct set of thiol groups are labelled at each concentration.

Protection experiments

The labelling experiments reported above indicate that thiol groups involved in some way with the generation of the E.O.G. response can be labelled. However, these thiol groups might have occurred on a variety of membrane proteins that were not directly involved with the sensing of odorants, but the activity of which were necessary for the ion-conductance changes involved in the receptor potential. To establish that the labelled thiol groups occur at the odorant-binding sites of the olfactory-receptor proteins, it is

Fig. 3. Time course of the effects of 1 mM-mersalyl in Ringer's solution, pH 7.0, on the E.O.G. responses to pentyl acetate and its subsequent reversibility by dithiothreitol (10 mM in Ringer's solution, pH 7.0)

---, After washing with Ringer's solution pH 7.0;
—, after treatment with dithiothreitol. Results are shown as means $\pm$ S.E.M. (for three frogs).

Fig. 4. Effect of pentyl acetate, in solution, on the decrease in the E.O.G. response to pentyl acetate vapour brought about by mersalyl

The results are expressed as the means $\pm$ S.E.M. for the numbers of animals given in parentheses. Symbols: $\circ$, 0.1 mM-mersalyl alone (18); $\square$, 0.1 mM-mersalyl and 0.1 mM pentyl acetate (4); $\bullet$, 0.1 mM-mersalyl and 1 mM-pentyl acetate (24); $\triangle$, 0.1 mM-mersalyl and 10 mM-pentyl acetate (1).

necessary to show that labelling of these groups can be prevented by using odorants that compete for the binding site containing the thiol group. When the odorant pentyl acetate was present in solution

in the olfactory cavity, before, during and after exposure to mersalyl, it protected the receptor sites from labelling by mersalyl (Fig. 4). When the olfactory mucosa was treated with pentyl acetate (1mm) in the absence of mersalyl for 12min and then washed, the peak amplitude of the E.O.G. response had decreased to $39 \pm 3\%$ (mean $\pm$ S.E.M. for five frogs) of the control E.O.G. response value. However, the E.O.G. response recovered with time and returned to the 100% value after 40min. The kinetics of this recovery were identical, within our experimental error, to those found for the same concentration of odorant in the presence of mersalyl (Fig. 4). Thus pentyl acetate at the concentrations used for the protection experiments affects the response of the olfactory neuron plasma membrane. The time course of the recovery probably reflects the time taken for desorption of the pentyl acetate from the tissue together with conformational rearrangements in the plasma membrane. There is only a small recovery of the E.O.G. response when the tissue is treated with mersalyl alone (Fig. 4). Increasing con-

centrations of pentyl acetate bring about an increasing amount of protection of labelling over the time period for which the experiment was run, and at high concentrations complete protection was obtained. If the mucosa that had recovered its full E.O.G. response in a protection experiment was treated for 2min with the same solution of pentyl acetate and mersalyl that had been used on it during the course of the protection experiment, the E.O.G. response was irreversibly decreased. Related experiments demonstrated that, for protection against labelling to be achieved, the tissue had to be treated with pentyl acetate for some minutes before the labelling was attempted.

Specificity of protection

The protection experiments show that pentyl acetate in solution can prevent abolition of the response to the same odorant in the vapour phase. The experiments do not indicate whether protection is a mechanism that preserves the response of the olfactory plasma membrane to all odorants, or

Fig. 5. Protection by pentyl acetate in solution of the E.O.G. responses to *n*-butyric acid, *n*-butyl acetate, phenylacetaldehyde and cineole, from the effects of mersalyl

The results are shown as the means $\pm$ S.E.M. for the numbers of animals given in parentheses. (a) $\circ$, Pentyl acetate (3); $\bullet$, butyric acid (3); (b) $\circ$, pentyl acetate (3); $\bullet$, butyl acetate (3); (c) $\circ$, pentyl acetate (2); $\bullet$, phenylacetaldehyde (2); (d) $\circ$, pentyl acetate (3); $\bullet$, cineole (3).

whether it specifically protects one or a limited class of sites. To distinguish between these possibilities we investigated whether protection by a solution of pentyl acetate would also preserve the response of other classes of odorants presented in the vapour phase. The results (Figs. 5 and 6; Table 1) show that pentyl acetate can protect some classes of odorants from labelling (Fig. 5), but fails to protect the sites at which other classes of odorants bind (Fig. 6). The results are summarized in Table 1. When the mucosa is treated with pentyl acetate in the absence of mersalyl and then washed, it recovers its E.O.G. response to each of the odorants shown in Table 1, with a time course similar to that found for recovery of the response to pentyl acetate vapour. Thus treating the mucosa with pentyl acetate solution does not abolish the activity of any of the sites that interact with the other odorants. The errors incurred in this type of experiment are large, as indicated by the error bars (Figs. 5 and 6). In the experiments to establish the specificity of the protection afforded by pentyl acetate, we have two conflicting experimental

requirements: the necessity to examine a representative range of odorants and the necessity to define the curves with sufficient precision so that we can ascertain whether or not protection has taken place. In general we have defined the curves with sufficient precision to distinguish between those cases in which protection had been achieved (Fig. 5) and those in which it was not achieved (Fig. 6).

In all the protection experiments, the response to pentyl acetate was measured with the response to the particular odorant being studied: this ensured that the animal was responding normally. Pentyl acetate in solution protected the binding sites of the following odorants against labelling by mersalyl: *n*-butyric acid, *n*-butyl acetate, phenylacetaldehyde and cineole. The recovery curves for these odorants (Fig. 5) commence at the same percentage inhibition as the curve for the control odorant pentyl acetate and the general shape of the curve resembled that of the control curve. The final degree of recovery reached at the end of the experiment was in each case less than that of the control curve, but still showed substantial

Fig. 6. Lack of the protection of the E.O.G. responses to *n*-butanol, benzyl acetate, nitrobenzene and β -ionone, from the effects of mersalyl, by pentyl acetate solution

The results are shown as the means $\pm$ S.E.M. for the numbers of animals given in parentheses. (a) ○, Pentyl acetate (2); ●, butanol (2); (b) ○, pentyl acetate (2); ●, benzyl acetate (2); (c) ○, pentyl acetate (3); ●, nitrobenzene (3); (d) ○, pentyl acetate (4); ●, β -ionone (4).

Table 1. *Ability of pentyl acetate to protect odorant-binding sites against labelling by the thiol-specific reagent mersalyl*

The experiments were carried out as described in the Materials and Methods section. The results are expressed as the percentage of the original E.O.G. peak amplitude before any reagents were applied. The values recorded were those found 34 min after washing the olfactory mucosa with Ringer's solution after treatment of the mucosa with mersalyl (0.1 mM) and pentyl acetate (1 mM) by using the protocol described in the Materials and Methods section. The E.O.G. peak amplitude (% of original) immediately before washing was in the range 25–40%. The E.O.G. peak amplitude (% of original) found after washing for 34 min when the mucosa was treated with mersalyl alone (0.1 mM) was 38 ± 5 (mean $\pm$ S.E.M. for 18 experiments). Values are means $\pm$ S.E.M. for the numbers of experiments given in parentheses.

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

recovery. Pentyl acetate in solution failed to protect the binding sites of the following odorants: butan-1-ol, benzyl acetate, nitrobenzene, β -ionone and linalyl acetate. The recovery curves for these odorants commenced at the same percentage inhibition as the curve for the control odorant, except for benzyl acetate, which had a lower initial value. However, the recovery curves for these odorants showed no significant increase over the initial value (Fig. 6).

Discussion

The molecular mechanisms involved in coding and transduction in sensory cells have been little studied, except in the case of visual receptors, where a detailed mechanism for the transduction in retinal rods has emerged from biochemical studies (Stieve, 1974). In the case of olfaction, however, there has been little attempt to distinguish experimentally between the speculative models for the coding and transduction steps that have been put forward (Beidler, 1971; Poynder, 1974).

In the present study we report in more detail on experiments with a group-specific reagent, one of the three chemical-modification methods that we have used on the olfactory system (Menevse, 1977; Menevse *et al.*, 1977a). The aim of the chemical-modification experiments is to identify the number of independent olfactory receptor proteins and to characterize the specificity of odorant binding to these proteins. These objectives can be attained by the specific inactivation of certain of the olfactory-receptor proteins and retention of the activity of others. Affinity labelling and photo-affinity labelling, with, in both cases, odorants in the vapour phase, are the preferred chemical-modification methods

when specific labelling of olfactory-receptor sites are required (Menevse, 1977; Menevse *et al.*, 1977a). Group-specific reagents can be used to label one site in a protein if there is a uniquely reactive functional group at the binding site. Getchell & Gesteland (1972) by using the thiol-specific reagent *N*-ethylmaleimide were able to inactivate odorant sites. This inactivation could be prevented if high concentrations (12.5 mM) of ethyl n-butyrate were used as a protecting agent. We have investigated the thiol groups on the olfactory mucosa by using the non-penetrant reagent mersalyl. In previous work (Menevse *et al.*, 1977a) we found that we could label groups on the olfactory mucosa with group-specific reagents, including *N*-ethylmaleimide in the vapour phase. However, since *N*-ethylmaleimide and other neutral reagents can penetrate membranes (Vignais & Vignais, 1973) and label intracellular proteins, it is important in investigating the olfactory receptor proteins to use non-penetrant reagents that label on the outside of the plasma membrane only; otherwise erroneous conclusions may be reached about the position of the odorant sites that have been labelled in the olfactory mucosa. The differing behaviour of penetrant and non-penetrant compounds is clearly seen in studies of the E.O.G. responses with cyclic AMP and dibutyl cyclic AMP (Menevse *et al.*, 1977b). Mersalyl has been clearly shown to be a non-penetrant reagent (Mavrier & Hanoune, 1975).

In our experiments we used a slightly lower concentration of the thiol-specific reagent than that used by Getchell & Gesteland (1972), and mersalyl appeared to be slightly more effective in decreasing the amplitude of the E.O.G. response. Under the conditions used, we were able to achieve 100% recovery of the E.O.G. response by using a pentyl

acetate solution in the olfactory cavity that was over an order of magnitude lower than the protecting solution of ethyl *n*-butyrate used in the *N*-ethyl-maleimide studies (Getchell & Gesteland, 1972).

The crucial question for our results is whether the protection effects we have observed indicate the presence of specific binding sites on an olfactory receptor protein or whether we have observed only a non-specific membrane effect. In the case of specific sites we would expect to see a correlation between the structure and odour-type of the odorants and their efficacy as protecting agents, analogous to the behaviour shown by competitive agonists and competitive inhibitors (Webb, 1963). If there were a well-defined specificity in the interaction of the pentyl acetate in solution with the binding sites on a receptor protein for fruity odorants, then odorants of a similar molecular type and odour would be expected to have their sites protected, whereas sites for odorants of a different molecular type and odour would not be expected to be protected. This would be the case if the quality-coding mechanisms operating in the initial odorant-receptor interaction were simple, in the sense that each odorant was binding to one type of receptor protein only. Since it is thought from studies on specific anosmia that there may be as many as 30 different types of olfactory receptor proteins, it is clearly a major task to characterize fully the specificity shown in experiments of the type carried out here. Our results are incomplete (Table 1), but clearly show that pentyl acetate in solution can protect the binding sites for some odorants with an unrelated structure and odour. As would be expected for the case of pentyl acetate binding to a site that specifically recognizes the structural features of this molecule, pentyl acetate is more effective at protecting its own binding sites than that of any of the other molecules examined. Moreover, this protection can be observed at concentrations of pentyl acetate (the protecting odorant) that are over two orders of magnitude lower than the concentration of ethyl *n*-butyrate that was used by Getchell & Gesteland (1972). High concentrations of the protecting odorant fully protected the binding sites for the vapour-phase pentyl acetate. The related odorant *n*-butyl acetate had its sites relatively well protected. Surprisingly, the structurally unrelated odorants cineole, phenylacetaldehyde and *n*-butyric acid have their binding sites protected to approximately the same degree. It is noteworthy that the three odorants pentyl acetate, *n*-butyl acetate and cineole consistently gave large highly reproducible E.O.G. responses. Perhaps this reflects a high density of receptors for these odorants on the frog olfactory membrane, with the receptor proteins for the camphoraceous odorant being adjacent in the membrane to those for the fruity odorants. Butyric acid belongs to a set of odorants, the fatty

acids, for which there is a well-defined specific anosmia (Amoore, 1970) indicative of a specific binding site for these odorants. If the acyl chain of butyric acid binds to a hydrophobic pocket in the binding site on the receptor protein, this pocket may be accessible to the acyl or alkyl chain of pentyl acetate. Such partial overlap of the two binding sites for two odorants would account for the observed protection. Comparable binding phenomena have been observed with hydrophobic sites on an allosteric enzyme (Dodd & Radda, 1969). A similar explanation may be given for the protective effect observed with phenylacetaldehyde, although the underlying structural similarities between the two odorants are less clear.

The remaining five odorants examined, butan-1-ol, nitrobenzene, benzyl acetate, β -ionone and linalyl acetate, did not have their binding sites protected by the solution of pentyl acetate (Table 1). If some of the other class of odorants mentioned above have a hydrophobic pocket at their binding sites that can be occupied by pentyl acetate, it is not clear why similar considerations do not apply to some of these odorants, especially butan-1-ol and benzyl acetate.

As an alternative to the above explanation, which invokes the concept of specific receptor proteins, the results may also be explained by invoking other mechanisms. The high concentrations of pentyl acetate solution presented to the neurons may affect the olfactory membranes in the following ways. The cells may be fully depolarized by the abnormally high stimulus concentration and this may affect the adaptation kinetics, thus explaining the slow recovery of the E.O.G. process. The fact that the kinetics of recovery of the E.O.G. after treatment of the mucosa with pentyl acetate alone are identical for all the odorants supports this interpretation. Alternatively, the pentyl acetate may bind non-specifically to the phospholipid phase in the membrane and thereby alter many of the enzymic and transport functions of the membrane through an expansion mechanism, as has been found in membrane stabilization (Seeman, 1972). The pentyl acetate might also directly cause a conformational change in the membrane proteins, including the ionophoric proteins. If any of these mechanisms were operating, it is less likely that we would have observed the differential protection effects obtained with the odorants. If a slow adaptation after depolarization of the neurons were taking place we would expect the cells to respond fully to all of the odorants when the full response to pentyl acetate had been obtained. Membrane stabilization by lipophilic ligands is readily reversible at low ligand concentrations and would not explain the differential protection found. High ligand concentrations can lead to irreversible membrane damage, which would manifest itself as a loss of the E.O.G. response

for all the odorants. Another possible mechanism might invoke differential sensitivity of the olfactory-receptor protein towards organic solvents. Membrane proteins can be selectively extracted from the membrane by detergents and organic solvents (Quinn, 1976; Austin *et al.*, 1974). The concentration (1mM) of pentyl acetate used in the protecting experiments, although considerably lower than the 12.5mM solution of ethyl butyrate used in the *N*-ethylmaleimide experiments (Getchell & Gesteland, 1972), may still act as a solvent in susceptible domains of the olfactory membranes, and may have inactivated the receptor proteins for the odorants whose sites were not protected by pentyl acetate.

On balance we think that the evidence favours the site-competition mechanism, but it should be noted that, should the other cited mechanisms be operating, the experiments would still provide evidence for the existence of classes of olfactory-receptor proteins.

We thank the Medical Research Council for financial support and The Royal Society for an equipment grant.

References

- Amoore, J. (1970) *The Molecular Basis of Odor*, Thomas, Springfield
- Austin, P., Dodd, G. H. & Leslie, R. (1974) *Biochem. Soc. Trans.* **2**, 963–964
- Barber, R. (1976) M.Sc. Thesis, University of Warwick
- Barratt, M. D., Dodd, G. H. & Chapman, D. (1969) *Biochim. Biophys. Acta* **194**, 600–602
- Beidler, L. M. (ed.) (1971) *Handbook of Sensory Physiology*, vol. 4, Springer-Verlag, Berlin
- Bostock, H. & Poynder, T. M. (1972) *J. Physiol. (London)* **224**, 14P
- Brocklehurst, J., Dodd, G. H., Freedman, R. B., Malcolm, A. B., Price, N. & Radda, G. K. (1970) in *Pyridine Nucleotide Dependent Dehydrogenases* (Sund, H., ed.), pp. 257–267, Springer-Verlag, Berlin
- Dodd, G. H. (1976) *Structure-Activity Relationships in Chemoreception* (Benz, G., ed.), pp. 55–61, Information Retrieval Ltd., London
- Dodd, G. H. & Radda, G. K. (1969) *Biochem. J.* **114**, 407–417
- Dodd, G. H., Menevse, A. & Poynder, T. M. (1978) *Abstr. Int. Symp. Olfaction and Taste 6th*, p. 59, Information Retrieval Ltd., London
- Doughty, M. K. & Dodd, G. H. (1978) *Comp. Biochem. Physiol. C* **59**, 21–32
- Frazier, J. L. & Heitz, J. R. (1975) *Chem. Senses Flavor* **1**, 271–281
- Gesteland, R. C. (1976) in *Structure-Activity Relationships in Chemoreception* (Benz, G., ed.), pp. 161–168, Information Retrieval Ltd., London
- Getchell, M. L. & Gesteland, R. C. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1494–1498
- Koshland, D. E., Jr. (1970) *Enzymes 2nd Ed.* **1**, 342–396
- Mavier, P. & Hanoune, J. (1975) *Eur. J. Biochem.* **59**, 593–599
- Menevse, A. (1977) Ph.D. Thesis, University of Warwick
- Menevse, A., Dodd, G. H., Poynder, T. & Squirrell, D. (1977a) *Biochem. Soc. Trans.* **5**, 191–194
- Menevse, A., Dodd, G. H. & Poynder, T. M. (1977b) *Biochem. Biophys. Res. Commun.* **77**, 671–677
- Ottoson, D. (1956) *Acta Physiol. Scand. Suppl.* **122**, 35, 1–83
- Poynder, T. M. (ed.) (1974) *Transduction Mechanisms in Chemoreception*, pp. 241–249, Information Retrieval Ltd., London
- Quinn, P. J. (1976) *The Molecular Biology of Cell Membranes*, p. 16, Macmillan Press, London
- Seeman, P. (1972) *Pharmacol. Rev.* **24**, 583–655
- Shaw, E. (1970) *Enzymes 2nd Ed.* **1**, 91–147
- Stieve, H. (1974) in *Biochemistry of Sensory Functions* (Jaenicke, L., ed.), pp. 79–107, Springer-Verlag, Berlin
- Tinberg, H. & Packer, L. (1976) in *The Enzymes of Biological Membranes* vol. 1, pp. 171–198, Plenum Press, New York and London
- Vignais, P. M. & Vignais, P. V. (1973) *Biochim. Biophys. Acta* **325**, 367–374
- Villet, R. H. (1974) *Nature (London)* **248**, 707–709
- Webb, J. L. (1963) *Enzyme and Metabolic Inhibitors* vol. 1, ch. 4, pp. 111–148, Academic Press, New York and London