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MOLECULAR MECHANISMS OF ODOR-SENSING

II. STUDIES OF FRACTIONS FROM OLFACTORY TISSUE SCRAPINGS CAPABLE OF SENSITIZING ARTIFICIAL LIPID MEMBRANES TO ACTION OF ODORANTS

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Summary

From the preparation obtained by ultrasonic disintegration of frog olfactory epithelium scraping, a fraction capable of sensitizing artificial phospholipid membranes to the action of some chemicals assumed to be odorants in frogs was isolated. In the presence of the active fraction, the membranes respond to the addition of camphor, linalool and musk ambrette by an increased permeability to Na^+ and Ca^{2+}. The main component of the active fraction is a nucleoprotein with molecular weight of no less than 100 000.

Introduction

Studies carried out in our laboratory have demonstrated that the preparation obtained by ultrasonic treatment of frog olfactory epithelium scraping contains a component capable of sensitizing artificial phospholipid membranes to the action of camphor, linalool, musk ambrette and some other chemicals [1]. These chemicals are assumed to be odorants in frogs [2], and here after will be referred to simply as "odorants". The ability of the membranes to respond to any of the above odorants is retained even after the response to any of the others has reached saturation. From these data, it could be supposed that the modifying agent contained in the frog olfactory preparation contributes to the process of olfactory transduction. To illucidate the role of this agent, it is necessary to isolate it and to determine its localisation within the olfactory tissue.

In the present study, the olfactory tissue preparation was fractionated by column chromatography on DEAE-Sephadex A-25. The fractions thus obtained were tested for their ability to sensitize artificial lipid membranes to the action of camphor, linalool and musk ambrette using the techniques developed for

unpurified preparations [1]. In addition, some biochemical properties of the active fraction were investigated.

Materials and Methods

Materials. Sephadex A-25, Sephadex G-50, Sepharose 6B from Pharmacia, Sweden; Millipore filters PSAC and PSJM from Millipore Corporation, U.S.A.; cellulose MN-300 from Nachery, Nagel, G.F.R.; sodium dodecyl sulphate and pepsin from Merck, Munchen, G.F.R.; bovine albumin from Reanal, Hungary; ovalbumin from Olayn Works, U.S.S.R.; adenine, cytosine, guanine and uracyl from Chemapol, Czechoslovakia; camphor from a Moscow pharmaceutical factory; musk ambrette and linalool were perfumery preparations. Camphor was recrystallized from methanol, while musk ambrette and linalool were used as received.

Derivation of olfactory preparation. To obtain the olfactory preparation, frogs *Rana temporaria* of either sex were used. The surface of the olfactory epithelium was carefully scraped by a scalpel blade under a light microscope. Commonly the scrapings from 100 frogs were suspended in 70 ml of 10 mM Tris · HCl buffer (pH 7.0). The suspended scrapings were then sonicated at 22 kcycles for 10—15 min at 0° C and centrifuged for 1 h at 40 000 × *g*. The clear, slightly yellowish supernatant thus obtained was used for modification and chromatography and was termed "crude olfactory preparation".

Formation of artificial lipid membranes respondsive to odorants. The attempts to make artificial membranes respondsive to odorants by mixing the olfactory preparation with brain lipid extract were of no success. Since the active material might be denaturated in the organic solvent, we modified the membranes by adding the olfactory preparation to the solution bathing the membrane. With such a modification technique, practically all the material remained in solution, as was verified by the absorption spectra. Thus, only a minor part of the material penetrated the membrane.

To form artificial membranes, we used a decane solution of brain lipid extract obtained using the method of Folch et al. [3]. The membranes were formed by the method of Mueller et al. [4] on a hole (1 mm in diameter) in 2 ml teflon cup placed in a glass chamber (20 ml in volume).

The addition of olfactory preparation to the cup after the membrane has formed creates a pressure difference between the cup and the chamber, and subsequent addition of odorous solution further increases the pressure difference, thus leading to a lower membrane stability. Therefore in the experiments reported below, the active preparation (0.5 ml) was added to the cup prior to membrane formation, while saturated aqueous solutions of odorants (0.5 ml) were added to the glass chamber.

Preparation of active fractions capable of sensitising artificial lipid membranes to the action of odorants. Step 1 (first ion-exchange). The crude olfactory preparation (100 ml) containing 50 mg of protein was applied to a column (2 × 25 cm) with DEAE-Sephadex A-25 which had been equilibrated with 10 mM Tris · HCl buffer (pH 7.0), containing 150 mM of sucrose. Elution was carried out using 600 ml of a solution with gradient from 10 mM Tris · HCl (pH 7.0)/150 mM sucrose buffer to the same buffer containing 0.6 M NaCl, at a

flow rate 40 ml/h. 3 ml fractions were collected. The criterion for activity of a fraction at each step of separation was its ability to sensitize the artificial lipid membrane to the action of odorants. Each fraction was tested separately for camphor, musk and linalool. Final concentrations of these odorants in the solution bathing the membrane were 10^{-4} M for camphor and 1/40 of saturation concentration for musk and linalool. A fraction was assumed to be active when the resistance of the sensitized membrane to the action of odorants decreased by at least a factor of five.

Step 2 (gel filtration). The active fractions resulting from the first step were combined, concentrated by ultrafiltration and applied to a column (3 × 60 cm) of Sephadex G-50 equilibrated with 10 mM Tris · HCl (pH 7.0) buffer containing 150 mM sucrose. The flow rate was 70 ml/h. Fractions containing high molecular weight components were collected and used for subsequent chromatography.

Step 3 (second ion-exchange, chromatography and concentrating). The fractions from Step 2 containing high molecular weight components were applied to the second DEAE-Sephadex A-25 column (2 × 25 cm), equilibrated and eluated as described above. The resulting active fractions were collected, chromatographed on Sephadex G-50 to remove NaCl (see Step 2) and concentrated by ultrafiltration on Millipore membrane filters.

Analytical determination. Protein was routinely determined by the method of Lowry et al. [5] using bovine serum albumin as standard. The absorption spectra were all measured with Specord UV-ViS double-beam spectrophotometer using quartz cells with light path of 1 cm.

Determination of molecular weight. The molecular weight was determined by chromatography on Sepharose 6B according to the method of Andrews [6]. Concentrated active fractions were incubated in 0.5% sodium dodecyl sulphate for 2 h at 37°C and then applied to a column (2 × 90 cm) which had been equilibrated with 50 mM sodium phosphate (pH 7.0) buffer containing 0.5% sodium dodecyl sulphate. The column was then eluted using the same buffer as above, at a flow rate of 40 ml/h. Bovine serum albumin (69 000), ovalbumin (43 000), pepsin (35 000) and myoglobin (17 000) were used as standards. The elution volumes of the above standards were plotted against the logarithm of their respective molecular weights. A molecular weights thus determined was compared to that found by gel electrophoresis (see the next paragraph).

Polyacrylamide gel electrophoresis. Gel electrophoresis was performed using Reanal equipment (Hungary) in one of the following buffer systems: 1, Tris · glycine buffer system, pH 8.9, polyacrylamide concentration 7.5% [7]; 2, 10 mM Tris · HCl buffer, pH 8,5, polyacrylamide concentration 7.5%; 3, 50 mM sodium phosphate buffer, containing 0.1% sodium dodecyl sulphate, polyacrylamide concentration 10% [8]. Samples of total volume 100 µl, containing 50 µg of protein and 50 µl of glycerol, were placed at the top of the gel. Bromphenol blue was used as a marker. Electrophoresis was performed at a constant current of 4 mA per gel.

In the case of buffer system 3, the samples were incubated in 0.1% sodium dodecyl sulphate and 0.1% mercaptoethanol for 2 h at 20°C prior to electrophoresis. The molecular weight markers used were bovine serum albumin, ovalbumin, pepsin and myoglobin.

The gels were stained with 0.25% Coomassie blue in 7% acetic acid for 10 h and destained with 7% acetic acid. When tested for nucleic acid, the gels were stained with 1% acridine orange in 2% lanthanum acetate for 10 h and destained in water [9].

Gels containing sodium dodecyl sulphate were stained with 0.2% methylene blue in solution containing 0.4 M sodium acetate and 0.4 M acetic acid [10]. In this case, destaining was done with water.

Determination of nucleic bases. The lyophylized active fraction (dry weight approx. 0.2 mg) was hydrolized with 0.5 ml 88% formic acid for 30 min in a sealed Pyrex tube, at 175°C [11]. After evaporation of the hydrolysate, the residue was dissolved in 50 µl of 1 M HCl and analysed using thin-layer chromatography on cellulose Mn-300 in an isopropranol/2 M HCl (65 : 35) system. The spots were visualized in ultraviolet light. Guanine, adenine, cytosine and uracil were taken as standards.

Results

The crude olfactory preparation, capable of sensitizing artificial lipid membranes, contained 90—95% of the total protein present in the scrapings. The protein concentration in the crude olfactory preparation was 1.4 mg/ml. It retained its activity for 4—7 days when stored at 4°C. The absorption spectrum of the preparation had a maximum at 262 nm (Fig. 1).

Assuming the modifying agent to be water-soluble, the ion-exchange chromatography was carried out without detergents. When, however, the eluating buffers containing no sucrose, we failed to obtaine fractions capable of modifying the membranes. Considering the ability of sucrose to prevent denaturation of protein [12] and its dissociating effect [13], we used buffer solutions containing 0.15 M of sucrose in all subsequent chromatographies. In this manner it was possible to obtain fractions capable of sensitizing the lipid membranes,

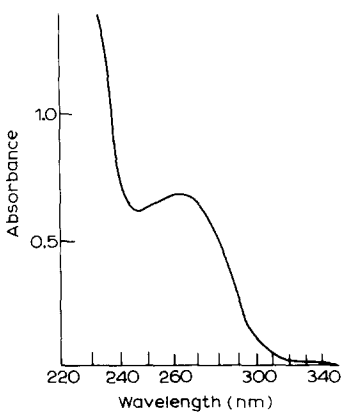


Fig. 1. Absorption spectrum of crude olfactory preparation (5-fold dilution).

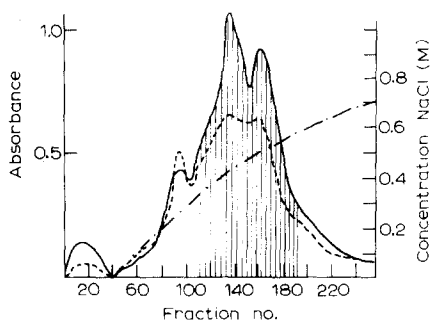


Fig. 2. DEAE-Sephadex A-25 chromatography of crude olfactory preparation. ———, absorbance at 260 nm; - - - - -, absorbance at 280 nm; — · — ·, NaCl concentration. The cross-hatched region corresponds to the active fractions.

though having chromatograms similar to those found in buffer systems without sucrose. The elution profile of the crude preparations is shown in Fig. 2. The active fractions had an absorption maximum at 260 nm. It should be noted that the membrane modification by active fractions was observed over a narrow pH range (7.35—7.45), as was the case with the crude olfactory preparation [1]. The lowering of modified membrane resistance to the addition of camphor, musk and linalool was observed only in media containing NaCl (Figs. 3b,c) or $CaCl_2$. The effect was not observed in media which contained KCl (Fig. 3a) or LiCl. $HgCl_2$ in 0.5 mM concentration blocked the response to all odorants tested, when added to the chamber both before and after the membrane modification. At the same time, $HgCl_2$ did not change the membrane resistance after the membrane had responded to odorants. Fig. 4 illustrates the effect of $HgCl_2$ on the membrane response to camphor.

The results presented here are similar to those obtained when the membrane was modified with crude olfactory preparation [1] and suggest that the modifying agent of the active fractions is the same as in the cruce preparation.

As seen from Fig. 2, peak 1 and peak 2 both contain the active fractions. The ratio of maxima of these peaks varied in different experiments from 2—0.8. At pH 10.0 (the pH value of eluent), the fractions of peak 1 and peak 2

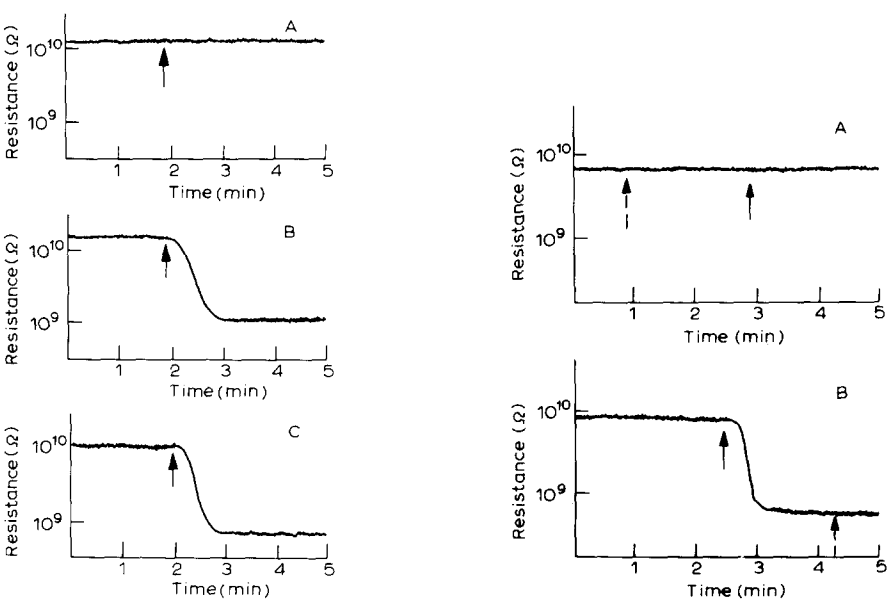


Fig. 3. Responses of the membranes modified by the active fractions, obtained by chromatography on DEAE-Sephadex, to camphor in media with different salt contents. The addition of camphor to the glass chamber indicated by arrows. 0.05 M Tris · HCl buffer (pH 7.4), containing 80 mM NaCl (A), 80 mM KCl (B), 40 mM NaCl and 40 mM KCl (C).

Fig. 4. Effect of $HgCl_2$ on the response of the membrane, modified by the active fractions, to camphor. 0.05 M Tris · HCl buffer (pH 7.4), containing 40 mM NaCl and 40 mM KCl. A, $HgCl_2$ is added to the cup prior to the addition of camphor; B, $HgCl_2$ is added to the cup after the addition of camphor. Final $HgCl_2$ concentration in the solution 0.5 mM. Full line arrow indicates the addition of camphor, broken line arrow indicates the addition of $HgCl_2$.

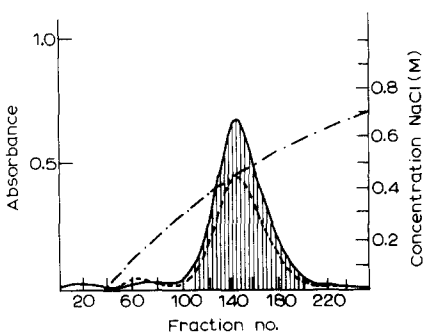
had similar absorption spectra. The chromatography of the combined fractions of peak 1 and peak 2 on Sephadex G-50 showed that both peaks contained two major components, i.e. a component with molecular weight less than 1500 and a high molecular weight component. The low molecular weight component absorption maximum shifted from 260 nm at pH 11.6 to 250 nm at pH 6.8. The absorbance of that component did not change on boiling for an hour.

The experiments showed that the ability of the high molecular weight component alone to modify the membrane did not depend on the presence of low molecular weight components.

Further purification of a modifying agent was carried out using rechromatography of the high molecular weight substance from Step 2 on DEAE-Sephadex A-25 (Step 3, Fig. 5). The absorption spectrum of active fractions capable of sensitizing the membrane is given in Fig. 6. The spectrum did not change after incubation of the fractions with 1% solution of sodium dodecyl sulphate or 8 M urea. The protein content of the rechromatographed preparation was 0.17 mg/ml, corresponding to an absorbance of 1.0 at 260 nm. The purification procedure is summarized in Table I.

The rechromatographed preparation was dialyzed to remove NaCl and concentrated by ultrafiltration through Millipore filters PSJM to an absorbance of 1.5—2.0 at 260 nm. Concentrating the preparation to an absorbance value higher than 3.0 resulted in aggregation of the molecules and thus to turbidity of the solution. The aggregation increased at pH lower than 5.7. The fact that the preparation concentrated on the filter PSJM suggests that the modifying agent had a molecular volume of no less than the molecules of ordinary proteins having a molecular weight in the order of 100 000.

An attempt to check the homogeneity of the rechromatographed preparation by electrophoresis in polyacrylamide gel with buffer system 1 containing no detergents was unseccessful. Only one band was observed on the top of the gels. This is probably due to the aggregation of the molecules as a result of con-



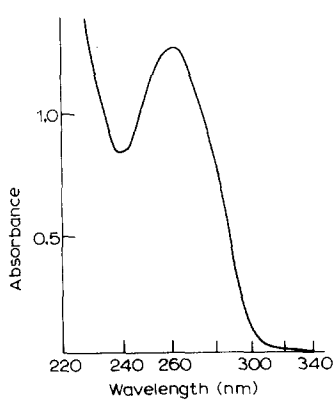


Fig. 5. Rechromatography of the active fractions on DEAE-Sephadex A-25. ———, absorbance at 260 nm; - - - - - -, absorbance at 280 nm; — · — ·, NaCl concentration. The cross-hatched region corresponds to the active fractions.

Fig. 6. Absorption spectrum of the active fractions of the rechromatographed olfactory preparation.

TABLE I

SCHEME OF PURIFICATION

Sample	Total protein content (mg)	Amount of protein necessary for modification (mg)	Purification (%)	Recovery (%)
Crude olfactory preparation	50	1.1	—	—
Rechromatographed fraction	0.4	0.055	20	15.2

centrating the preparation by electrophoresis. In buffer system 2 (Tris · HCl buffer, pH 8.5), a diffuse band stained with both Coomassie blue and acridine orange was observed (Fig. 7). Upon electrophoresis in the presence of sodium dodecyl sulphate, i.e., in system 3, two bands were observed, one stained with methylene blue and the other stained with Coomassie blue. In some experiments, after staining the gels with Coomassie blue, a secondary faintly stained band was observed. The presence of this secondary band was probably due to

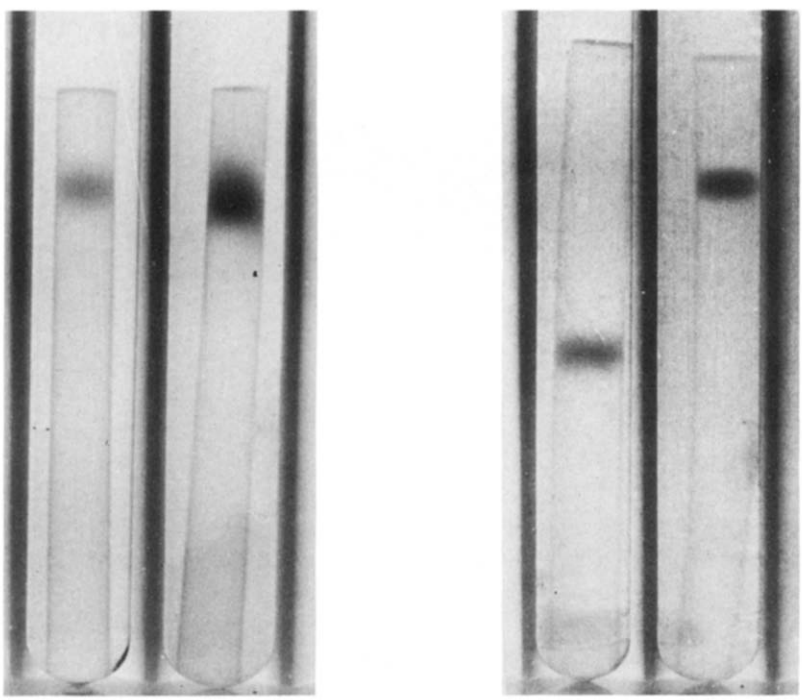
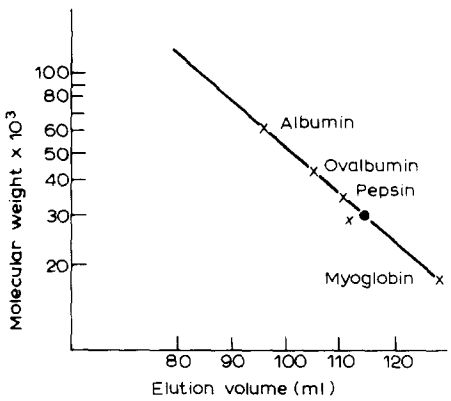


Fig. 7. Polyacrylamide gel electrophoresis of the rechromatographed olfactory preparation (buffer system 2). The gel on the left is stained with acridine orange, the gel on the right is stained with Coomassie blue.

Fig. 8. Polyacrylamide gel electrophoresis of the rechromatographed olfactory preparation in the presence of sodium dodecyl sulphate (buffer system 3). The gel on the left is stained with methylene blue, the gel on the right is stained with Coomassie blue.



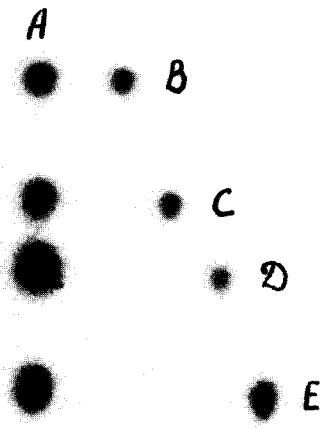


Fig. 9. Molecular weight determination of the nucleic moiety (x) of the nucleoprotein by gel filtration on Sepharose 6B in the presence of 0.5% sodium dodecyl sulphate.

Fig. 10. Thin-layer chromatography of the hydrolysate of the active fractions obtained by rechromatography on DEAE-Sephadex A-25. A, sample; B, uracyl; C, cytosine; D, adenine; E, guanine.

contamination in the rechormatographed preparation (Fig. 8). The molecular weight of the component stained with methylene blue was 30 000, while that of the component stained with Coomassie blue was 60 000—70 000.

From the electrophoresis studies, it could be supposed that the rechromatographed fraction contained a nucleoprotein which dissociated into nucleic acid and protein under the effect of sodium dodecyl sulphate. Additional evidence supporting this view was found by gel filtration of the rechromatographed fraction trough Sepharose 6B in the presence of sodium dodecyl sulphate. The molecular weight of the component having the absorption maximum at 260 nm was found to be 30 000, in good agreement with the above electrophoresis data (Fig. 9). The fact that the rechromatographed fraction contained nucleic acid was verified by thin-layer chromatography of its hydrolysate obtained using formic acid. Four bases characteristic for RNA were found (Fig. 10).

Discussion

Cherry et al. [14] reported variations in the resistance of artificial lipid membrane under the action of some chemicals. In the present experiments, however, no detectable change in the resistance of unmodified membranes was observed in response to camphor, musk and linalool. The resistance changed only in membranes modified by active fractions. It is therefore difficult to compare the present results to those of Cherry et al.

All data reported here strongly suggest that the main component of the active fractions, nucleoprotein, is the agent capable of sensitizing artificial membranes to the action of odorants. At the same time, the modification of a membrane may result from absorption of even few molecules of a modifying agent. This agent could be protein contained in minute conconcentrations in the active fraction and is thus undetectable by electrophoresis. This protein should be similar in its chromatographic properties to the nucleoprotein isolated in this study. According to the ultrafiltration data, this protein has a molecular weight of at least 100 000.

The problem of identification of the modifying agent present in frog olfactory preparation is similar to that encountered in the study on Excitability Induced Material by Mueller and Rudin [15]. Kushnir [16] has shown that the active agent in the latter case was also nucleoprotein. Methods used in the investigation of excitability induced material, namely, examination of the effect of ribonucleases on electrical characteristics of modified membranes [16], are applicable also in our case. They could be combined with studies on binding of radioactive odorants by active fractions, an approach that could not be applied to studies of the excitability induced material.

The concentration of nucleoprotein in the frog olfactory preparation is sufficiently high, such that nucleoprotein in combination with the low molecular weight component should determine the absorption spectrum of the crude olfactory preparation. Similar absorption spectra were recorded (Fesenko, E.E. and Peruvukhin, G.Ya., unpublished data) for crude olfactory preparation of pig and rat olfactory tissues, which might be determined also by nucleoprotein. "Chromoproteins" with the absorption maximum at 260 nm, isolated by Kurihara [17] from bovine, horse and dog olfactory tissues, are probably also nucleoproteins similar to that isolated in the present work.

The fact that the absorption spectrum of low molecular weight components did not change on boiling indicates that it is not determined by ascorbic acid, as previously suggested Ash [18]. Some of these components may be identical with the water-soluble pigments from the olfactory tissue isolated by Kurihara [17].

To obtain a preparation capable of modifying the membranes, we used rather hard treatment, namely ultrasonic disintegration. In other works, the modifying agent is incorporated into relatively rigid structures.

As reported in ref. 1, a low sensitivity of modified membranes is determined by a low value of equilibrium association constant of binding between odorants and modifying agents. This would suggest that the latter cannot be a receptor element of olfactory-cell membrane. The solubility of the modifying agent in aqueous solutions as shown in the present work is further evidence supporting

this view. It seems likely that in our experiments the artificial lipid membrane only played the part of a sensitive detector of the interaction between the modifying agents and odorants. On the other hand, the ability of a modifying agent to interact with different odorants may be indicative of its important role in odor sensing. It could be supposed that the modifying agent acts as an odor carrier transporting odorous molecules to membrane receptors of olfactory cells and removing them from the olfactory epithelium. The modifying agent may constitute part of a special transport system [19] which is destroyed by ultrasonic treatment. Further studies on the interaction between the modifying agent and odorants and the localization of the modifying agent within olfactory epithelium may elucidate the validity of the above hypothesis.

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