Biochemical Studies of Olfaction: Role of Cilia in Odorant Recognition[1]

LINDA D. RHEIN[2]

Abstract: Chemoreception in vertebrates is beginning to be understood. Numerous anatomical, behavioral, and physiological studies are now available. Current research efforts are examining the molecular basis of chemoreception. Rainbow trout (*Salmo gairdneri*) have a functional olfactory system and are a suitable vertebrate model for studying odorant interactions with receptors. Using a biochemical approach, initial events of olfactory recognition were examined; the aim was to determine the location and specificity of odor receptors. Cilia occupy the distal region of the receptor neuron on the trout olfactory epithelium, and their membranes are the postulated locus of odorant receptor sites. A cilia preparation was isolated from the olfactory rosette. The preparation was characterized by quantifying biochemical markers for cilia, along with electron microscopy, all of which substantiated enrichment of cilia. Functional activity was assessed by quantifying binding of several radioactively labeled odorant amino acids. The odorants bound to the cilia in a manner similar to the sedimentable preparation previously isolated from the olfactory rosette of the same animal, thus verifying the presence of odor receptors in the cilia preparation. Evidence also confirmed a site TSA which binds L-threonine, L-serine, and L-alanine and a site L which binds L-lysine (and L-arginine). Binding of L-serine and D-alanine showed evidence for a single affinity site while the others showed two affinity sites. Separation of membrane fractions from the cilia preparation revealed that binding activity is associated with a very low density membrane fraction B.

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Our knowledge of the sensory systems of vertebrates has been expanding rapidly. Chemosensory systems such as taste and olfaction are reasonably well defined anatomically. Behavioral and physiological studies have verified many gustatory and olfactory stimuli in a variety of vertebrate organisms. Moreover recent studies are now focusing on the molecular basis of olfactory and gustatory recognition. The present review will summarize results of one biochemical approach used to investigate olfactory recognition at the molecular level. These studies concentrate on the olfactory system of the salmonid fish because sufficient behavioral and physiological information is available on olfaction in these animals, verifying they have a functional olfactory system.

Salmonid fish have a well-developed olfactory system. They rely primarily on olfaction and vision during migration to their spawning waters (14). The rainbow trout (*Salmo gairdneri*) has been studied extensively and thus serves as a useful model for vertebrate olfaction. The morphology of the rainbow trout olfactory system is now described (27). In addition, electrophysiological and biochemical studies have verified amino acids as odorants to these animals (5,13). More recent efforts (25,26) were aimed at determining the location and specificity of odorant receptors for amino acids in the epithelium of the rainbow trout olfactory organ. The olfactory cilia, which are presumably the first part of the olfactory receptor neuron to contact odors, are the postulated locus of odorant receptors; the receptors are probably integral parts of the cilia membrane. Biochemical studies have provided experimental evidence for this hypothesis and will be discussed in detail.

CILIA MORPHOLOGY

The olfactory organ of rainbow trout is similar to that of other salmonid fish (35) consisting of a circular rosette, 4–6 mm in diameter, upon which are located 11–14 primary lamellae radially oriented around a medial depression (27). The sides of the lamellae are thrown into secondary folds resulting in parallel alternating ridges and depressions (Fig. 1A). The sensory olfactory epithelium is located in the depressions as verified by electrophysiological recordings of responses to odors (24, 32). In the distal part of the depressions, five cell types are distingushed (27). Two of these cell types contain cilia and a third type contains

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[2]Monell Chemical Senses Center, Philadelphia, Pennsylvania.

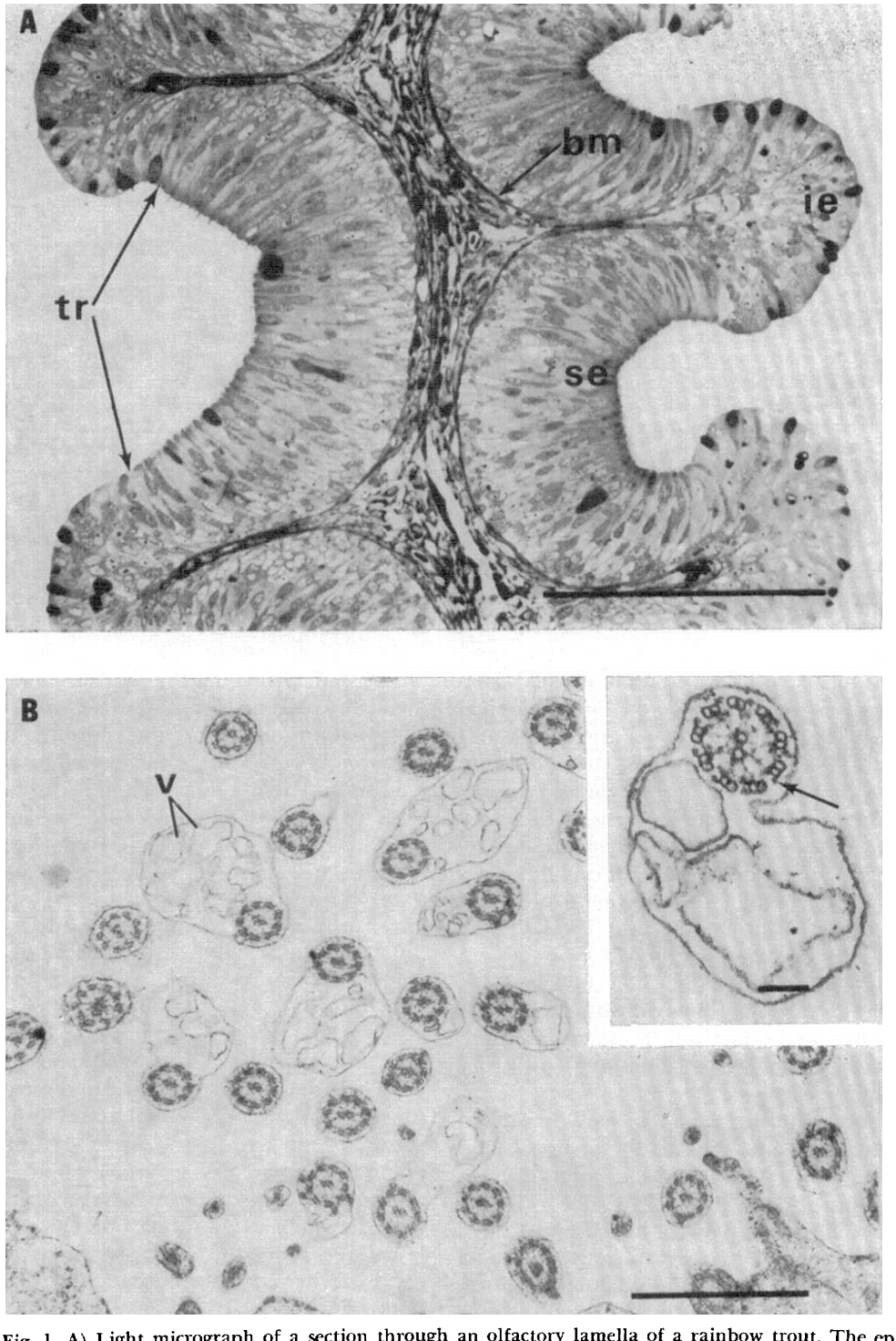

Fig. 1. A) Light micrograph of a section through an olfactory lamella of a rainbow trout. The epithelium and the basement membrane (bm) are thrown into folds resulting in a pattern of alternating ridges and depressions. The sensory epithelium (se) is located in the depressions, the indifferent epithelium (ie) occurs on the ridges and a distinct transition zone (tr) separates the two epithelia. × 400.

B) Transverse section of cilia in the olfactory mucosa of rainbow trout. All sections have the typical "9 + 2" microtubule fiber pattern. The plasma membrane often is expanded to include irregular vesicles (v). On the insert the arrows denote dynein arms on the outer doublet fibers. × 25,000 (insert × 70,000). Taken from Rhein et al. (27).

microvilli projecting from their apical surface. Receptor sites for odorants are thought to be found at the surface of these cell types. Moreover any researchers have discussed cilia as the initial site for odorant interactions (15,22) ,and our biochemical evidence supports this hypothesis (25,26).

Most sense organs contain cilia or modified cilia, and still others contain microvilli (2,6,10,21). The membrane covering these structures serves as the interface between the receptor cell and the environment and is the most likely candidate to contain the receptor molecules. This is well established for visual receptors. The photoreceptive molecules are located on disks that develop by complex invaginations of the membrane of a single cilium on the distal region of the rod receptor (1,7,9).

The cilia on the olfactory cells of trout contain the usual "9 + 2" complement of microtubule fibers (Fig. 1B) extending throughout the axoneme (12). Cross sections of cilia reveal dynein arms on the outer nine doublet fibers. These arms contain a Mg^{++} ATPase whose activity is important for cilia motility (30). In addition, the plasma membranes surrounding the cilia in trout are not uniformly tight sheaths but instead are often expanded to include smooth-walled irregular clear vesicles outside of the axoneme (Fig. 1B). Two types of cilia are seen: Type II cell cilia are located on a wide, flat surface and are usually several microns longer than Type I cell cilia which project from the base of a knob-like apical projection (27).

ROLE OF CILIA

Physiological evidence: The hypothesis that cilia are directly involved in the initial interaction with odorants appears reasonable but in fact has been controversial. In a preliminary report, Tucker (33) found that removal of cilia from turtle olfactory epithelium with detergent resulted in a preparation that continued to be electrophysiologically active upon chemical stimulation. Bronshtein and Minor (4), using frog olfactory epithelium, showed that brief exposure to the detergent triton X-100 destroyed the cilia. A concomitant decline in the EOG (electro-olfactogram) response to chemical stimuli (butylacetate) was observed. After a few days the cilia regenerated; during the regeneration period the EOG response was gradually restored.

In still other studies (8) based on odorant diffusion models, results suggest that the odorant must diffuse to the proximal region of the cilia before it interacts with a receptor site. A report by Blank et al. (3) revealed that application of odorants to frog olfactory epithelium increased ciliary activity and synchronized their movement.

Biochemical evidence: The rainbow trout olfactory system responds to a number of amino acids as odorants; the response is measured electrophysiologically from the olfactory bulb (13). Cagan and Zeiger (5) reported binding of radioactively labeled amino acids with a crude sedimentable preparation (Fraction P2) isolated by differential centrifugation from a trout olfactory rosette homogenate. The extent of binding of a series of amino acids paralleled their relative stimulatory effectiveness recorded in the bulb. This finding suggested that binding is a relevant measure of an early event in olfaction. In this regard, a similar sedimentable preparation isolated from the brain of rainbow trout bound L-[^{3}H]alanine at only 16% of the amount bound by Fraction P2 from the olfactory rosette. This control demonstrated the much higher level of binding activity with the olfactory preparation.

To assess biochemically the role of the olfactory cilia in the initial events of olfactory recognition, we isolated a functionally active cilia preparation from the trout rosettes (25,26). The cilia preparation was characterized morphologically and biochemically, using markers for cilia. The binding specificity of several amino acid odorants with the cilia was examined to assess functional activity.

Cilia were isolated by the method of Watson and Hopkins (34) and Linck (20). The procedure (Fig. 2) involves stirring whole rosettes in deciliation medium for about 20 min followed by differential centrifugation to obtain a cilia pellet (25). While only 0.5% of the whole rosette protein is recovered in the cilia preparation, approximately 50% of the total rosette binding activity for L-[^{3}H]alanine (method to be described later) is isolated with the

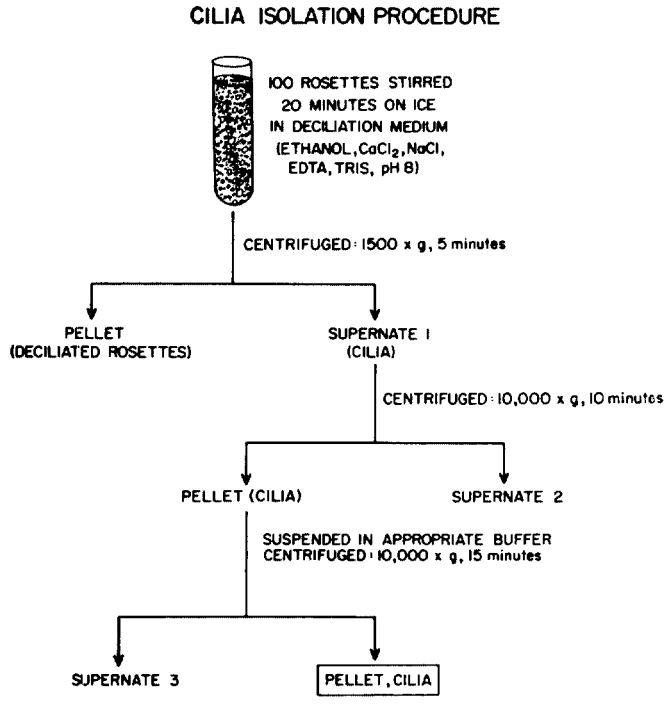

Fig. 2. Cilia isolation procedure. Cilia were isolated by a method used by Watson and Hopkins (34) to obtain tetrahymena cilia. Details are described previously (25).

cilia. This is encouraging and suggests that specific binding activity is enriched several fold in the cilia preparation compared to the rosette homogenate.

Enrichment of cilia in the isolated preparation was verified using morphological examination as well as with chemical and enzymatic markers for cilia (25). Two chemical markers were sought—the guanine: adenine ratio and the presence of the protein tubulin. The quantity of guanine relative to adenine is used because cilia microtubules contain 2 moles of bound guanine nucleotides (31) but essentially no adenine nucleotides. Substantial enrichment of guanine was found in the isolated cilia preparation (Table 1). In fact the ratio of guanine:adenine was 2.6-fold higher in the cilia preparation compared with whole rosettes and was 22-fold higher compared with deciliated rosettes. The biochemical marker provides evidence that the isolated preparation is enriched with cilia.

The microtubule fibers in the cilia axoneme contain the protein tubulin whose α and β subunits are separable by polyacrylamide gel electrophoresis (16,18). Electrophoretic analysis of the cilia preparation showed two bands that comigrated with authentic tubulin standards (25), again suggesting enrichment of cilia in the preparation.

An enzymatic marker, Mg^{++} ATPase (30) was also quantified in the isolated prepara-

Table 1. Nucleotide base content of the cilia preparation from trout olfactory rosettes

Sample	Nucleotide base content*				Ratio (Guanine:Adenine)
	μg/gm wet weight		μg/mg protein		
	Guanine	Adenine	Guanine	Adenine	
Whole rosettes	68.2	104	0.99	1.50	0.66
Deciliated rosettes	8.2	106	0.12	1.55	0.08
Cilia	0.35	0.20	1.31	0.76	1.73

*The values expressed relative to the wet weight refer to the original whole rosette sample. Those expressed relative to protein refer to the protein in that fraction. The results are from a single cilia preparation from 385 fish (770 rosettes). Taken from Rhein and Cagan (25).

tion (25). This enzyme comprises the dynein arms and is required for cilia motility. The marker was enriched several fold in the cilia compared with whole rosettes and decilated rosettes (Table 2), thus providing additional evidence for enrichment of cilia in the preparation. The three biochemical markers taken together, along with the observation by electron microscopic examination of cilia and ciliary fragments in the preparation (Fig. 3), support its enrichment with cilia.

ODORANT BINDING

Functional activity of the olfactory cilia preparation was assessed by quantifying binding of radioactively labeled odorant amino acids. The assay (25) involves incubating an aliquot of the cilia (in buffer, pH 7) with the radioactive amino acid for 60 min on ice. Duplicate aliquots are filtered using millipore filters and rinsed as described (5). The filter containing the attached cilia and its bound ligand is counted for radioactivity. This assay was originally used to study binding of taste stimuli to a sedimentable preparation from catfish taste tissue (17) and was later applied to a similar preparation (Fraction P2) from trout olfactory tissue (5).

Binding of ^{3}H-labeled L-threonine, L-serine, L-alanine and L-lysine and also of D-[^{14}C]alanine was examined. Results of binding studies showed that the preparation was active with respect to odorant recognition (Fig. 4). Binding was saturable and reversible. D-alanine was bound to a lesser extent than L-alanine. Scatchard (28) analysis (Table 3) of the data in Figure 4 showed evidence of two types of binding sites for L-threonine, L-alanine and L-lysine —a higher affinity binding site ($K_D \sim 10^{-6}$ M) and a lower affinity site ($K_D \sim 10^{-5}$M). For the odorants L-serine and D-alanine, there appeared to exist only one affinity binding site. These results paralleled those found by Cagan and Zeiger (5) for the sedimentable preparation (Fraction P2) isolated from the trout olfactory rosettes.

Recent competition studies (Rhein and Cagan, submitted for publication) revealed

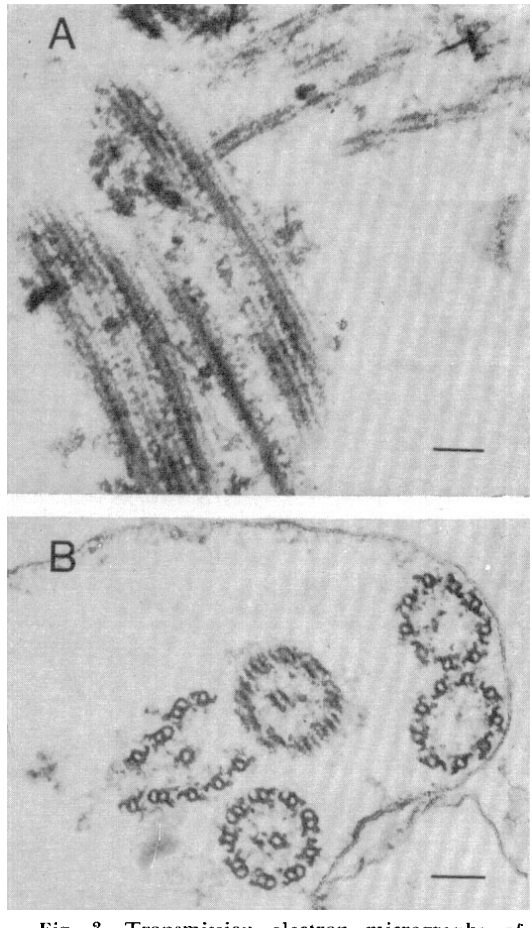

Fig. 3. Transmission electron micrographs of ciliary material in the isolated preparation from trout olfactory rosettes. Bar = 0.1 μm. Taken from Rhein and Cagan (25).

Table 2. Activity of Mg^{+2}-ATPase in the cilia preparation from trout olfactory rosettes*

Sample	Mg^{+2}-ATPase†	
	Total activity (μmol/min)	Specific activity (nmole/min-mg protein)
Whole rosettes	5.16 (100%)	73.9
Deciliated rosettes	4.12 (79.8%)	65.5
Supernatant	0.434 (8.4%)	44.4
Cilia	0.118 (2.3%)	275.0

*The values for total activity are expressed per gram of wet weight of the original rosette sample. In parentheses are shown the recoveries expressed as a percentage of the total activity of the whole rosette. The results for cilia are from a single preparation of 300 fish (600 rosettes). Taken from Rhein and Cagan (25).

†In Rhein and Cagan (25) the correct extinction coefficient was used in the calculations (6.22×10^3 M^{-1} cm^{-1}), but a typographical error in the paper showed it as $6.22 \times 10^{-3} M^{-1}$ cm^{-1}.

Table 3. Binding parameters of odorant amino acids with the cilia preparation from trout olfactory rosettes*

Amino acid	High affinity		Low affinity	
	K_D (μM)	B_{max} (pmole/mg)	K_D (μM)	B_{max} (pmole/mg)
L-threonine (n = 3)	1.6	140	35	480
L-serine (n = 5)	3.3	220	...	...
L-alanine (n = 5)	2.4	120	45	360
L-lysine (n = 1)	6.1	150	65	600
D-alanine (n = 2)	...	...	60	260

*The parameters were calculated from Scatchard plots of each individual preparation comprising the data for the binding curves shown in Fig. 6. The values of n are the numbers of preparations analyzed. Taken from Rhein and Cagan (25).

mutual competition among L-threonine, L-serine, and L-alanine for binding with a postulated site TSA on the cilia; the basic amino acids, L-lysine, and L-arginine competed for binding to a postulated site L but did not compete for site TSA to any appreciable extent. The two binding sites TSA and L were originally postulated by

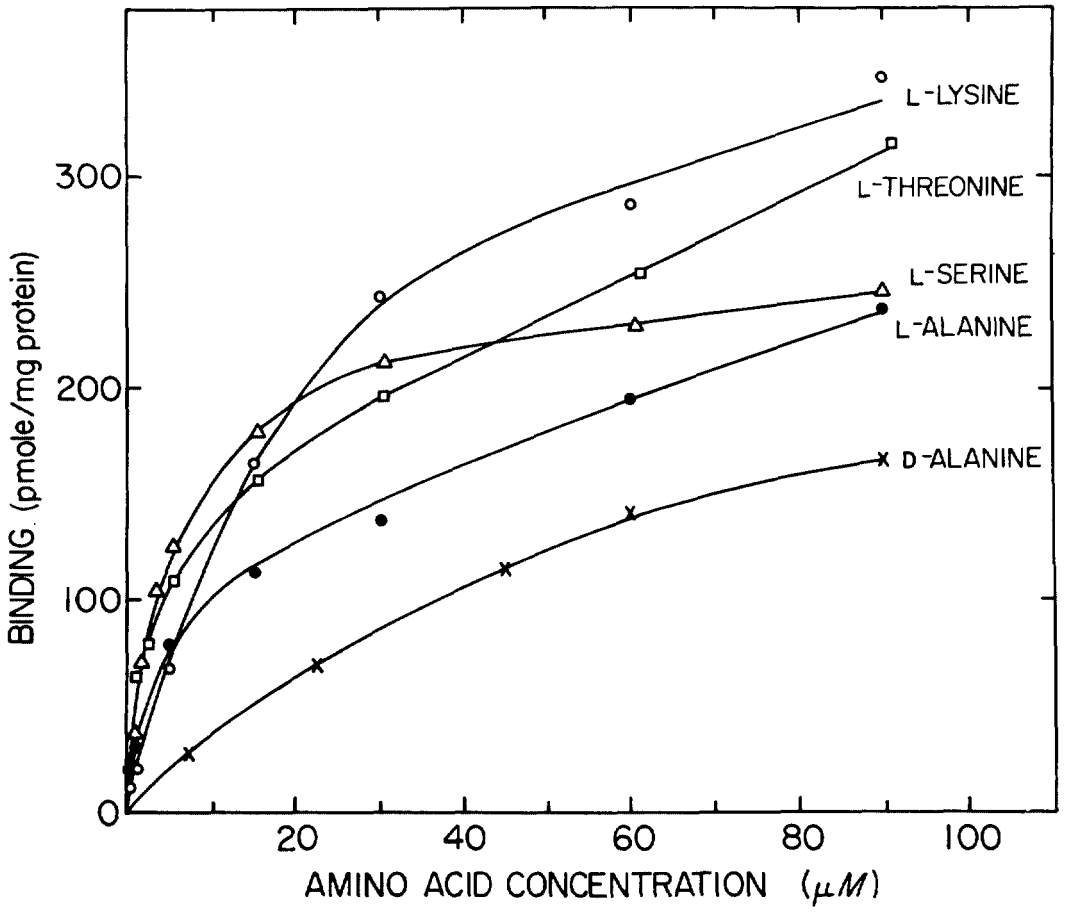

Fig. 4. Binding of odorant amino acids to the cilia preparation from rainbow trout olfactory rosettes. The data for each amino acid are the means of five preparations for L-alanine and L-serine, three for L-threonine, two for D-alanine, and one for L-lysine. The binding valve of 252 pmole/mg for D-alanine at 180μM is omitted from the curve for space purposes. Taken from Rhein and Cagan (25).

Cagan and Zeiger (5) from results of similar competition studies with the sedimentable preparation. The cilia preparation thus appears functionally active and we concluded, based on biochemical evidence, that the odorant receptors are associated with the cilia (25).

ODORANT RECEPTORS

Receptor sites for odorants are hypothesized to be located on cell membranes (19) at the surface of olfactory receptor neurons. It has further been suggested that the membranes of the cilia are the loci of odorant receptors (15,22,23). Membrane fractions were isolated from the cilia preparation and examined for odorant binding activity (26). The procedure involves separation of the homogenized cilia preparation by centrifugation at high speed on discontinuous sucrose density gradients as shown in Figure 5. Centrifugation conditions were chosen based on procedures used to separate plasma membranes (11) and central nervous system myelin fractions (29). The cilia samples were layered on top of the gradient, and after centrifugation five membrane fractions (A–D) and a pellet (P) were separated.

The odorants, L-[^{3}H]alanine and L-[^{3}H]lysine, were examined for binding activity with the resulting membrane fractions (Fig. 6). These odorants were chosen because they appear to interact at separate binding sites. The highest specific binding activity for both ligands was found in fraction B even though this fraction contained only a small amount of total protein in the preparation. This minor fraction was unique in that it was a very low density fraction. The low density suggests a high portion of lipid relative to protein, perhaps on the order of 75% lipid (see 29). More extensive research is necessary to characterize this fraction with respect to the lipid and protein components.

SUMMARY

To summarize, a successful biochemical approach was undertaken to begin examing the molecular basis of initial events in vertebrate olfactory chemoreception. The objective was to determine the localization of odorant receptor sites and to assess the specificity of odorant interactions with these sites. It is concluded (i) that the odorant receptor sites are associated with the membranes of the olfactory cilia, (ii) that odorant binding with these sites is an ini-

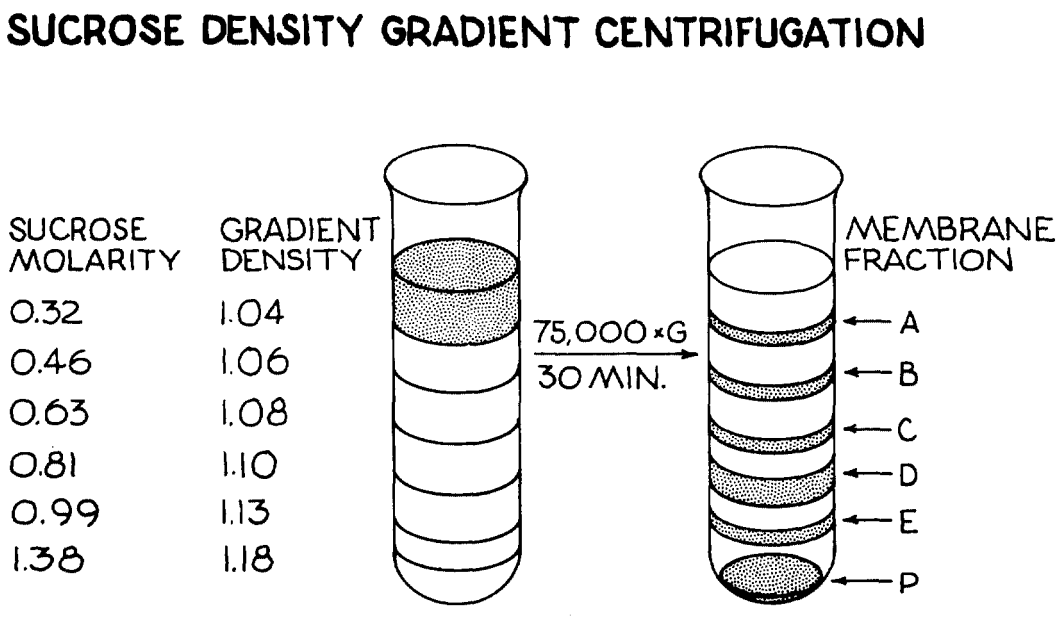

Fig. 5. Separation of cilia membranes using sucrose density gradient centrifugation. The cilia preparation was homogenized in 0.32 M sucrose on ice and applied to the top of the gradient as shown (shaded area). After centrifugation, 5 membrane fractions (A-E and a pellet (p) were separated.

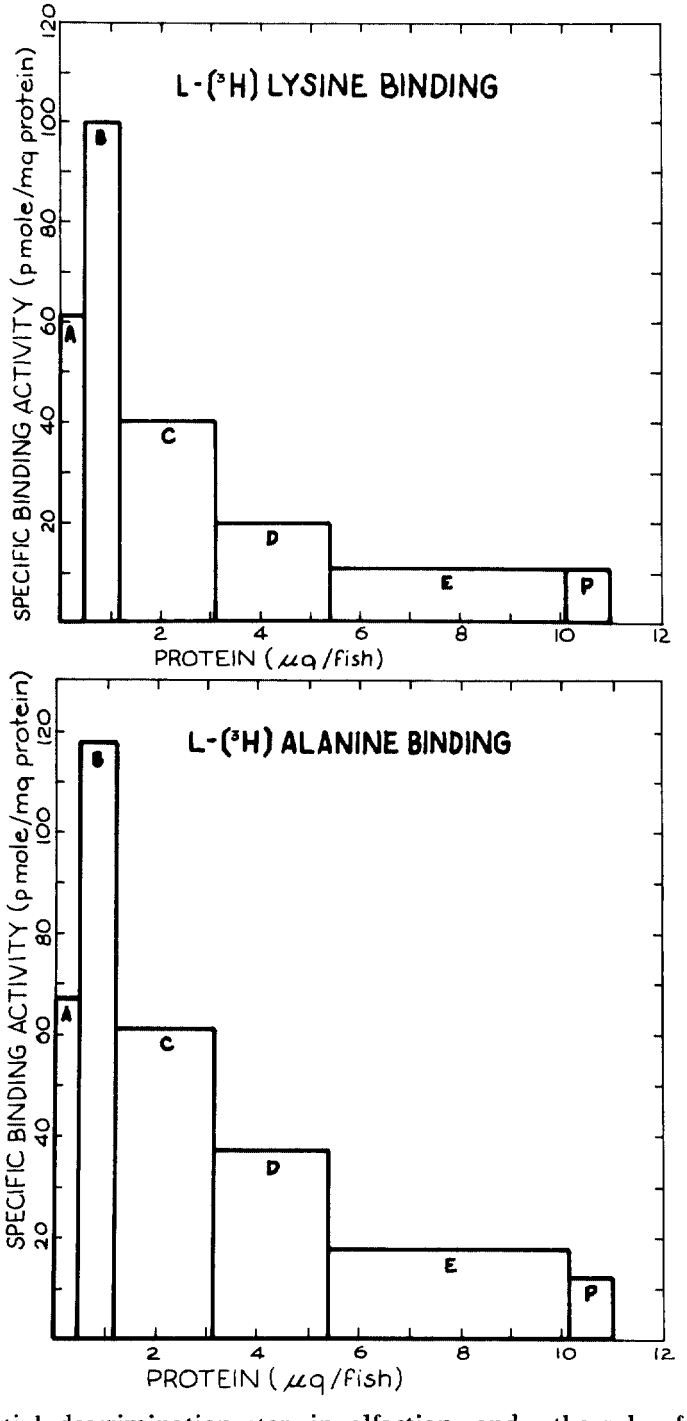

Fig. 6. Binding of radioactively labeled odorants to membrane fractions from trout olfactory cilia. The odorants L-[³H] lysine (top) and L-[³H] alanine (bottom) were examined because they represent two different populations of receptors. The values are for a single preparation from 250 fish. Radioactive ligands were at 0.5 μM.

tial descrimination step in olfaction, and (iii) that receptor sites exist with differing specificities for olfactory stimuli.

Future research in vertebrate olfactory chemosensation will involve characterization of the cilia membrane fractions. Information may help understand and predict the role of various membrane components in binding with odorants and in transducing binding information. Additionally, identification and characterization of odorant receptor molecules will be of foremost interest. This will include determining the number of different kinds of receptor mole-

cules and their specificity. In addition, chemical characterization of the odorant binding sites will be an important issue. Technology is available for answering these questions, and the findings should prove exciting.

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