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Biochemistry

Biochemical studies of olfaction: Isolation, characterization, and odorant binding activity of cilia from rainbow trout olfactory rosettes*

(olfactory cilia/binding sites/olfactory receptors)

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ABSTRACT **The role of cilia in recognition of olfactory stimuli has been controversial. Cilia from the intact olfactory rosettes of the rainbow trout *Salmo gairdneri* were isolated, characterized biochemically, and examined by electron microscopy. The markers studied are those associated with cilia in other organisms. Dynein arms contain Mg^{2+}-ATPase; this enzyme was enriched in the isolated cilia preparation. Guanine nucleotides are associated with the outer microtubule doublets of cilia but adenine nucleotides are not; a substantial enrichment in guanine, relative to adenine, was found in the cilia preparation. Tubulin, the structural protein component of microtubules, occurs in large amounts in cilia. Disc gel electrophoresis indicated tubulin in the cilia preparation. Electron microscopy confirmed the presence of cilia in the isolated preparation. Rainbow trout have an acute sense of smell and many amino acids are odorants to this species. Functional activity of the cilia preparation relevant to odorant recognition was assessed by using binding of radioactively labeled odorant amino acids. L-Alanine, L-serine, L-threonine, L-lysine, and D-alanine bound to the cilia preparation. This study provides direct biochemical evidence that olfactory cilia bind odorant molecules and supports the hypothesis that odorant recognition sites are integral parts of the cilia.**

The biochemical basis of odorant recognition is beginning to be understood. The numerous hypotheses proposed to explain odor specificity (1) are generally based upon structure–activity correlations made either psychologically in human subjects or electrophysiologically in various animal species. The rainbow trout *Salmo gairdneri*, which has a functional olfactory system, provides a suitable biochemical model for studying the specificity of odorant interactions with receptor sites. Their olfactory receptors are sensitive to amino acids as stimuli (2, 3). Amino acids bind specifically to a sedimentable fraction isolated from rainbow trout olfactory tissue (4). The extent of binding corresponds to the stimulatory effectiveness recorded electrophysiologically (3) from the olfactory bulb of the brain.

The hypothesis that olfactory cilia are the loci of olfactory receptor sites had been postulated for many years (5, 6) but has remained controversial. A widely cited preliminary report (7) described results suggesting that removal of cilia, by use of detergents, from turtle olfactory epithelium resulted in a preparation that continued to be electrophysiologically active. Earlier experiments by Shibuya (8) were unclear regarding involvement of cilia, but recent results described by Bronshtein and Minor (9) support the hypothesis. They showed that, following removal of frog olfactory cilia with detergent, the electroolfactogram responses to chemical stimulation declined. Upon regeneration of cilia, observed by electron microscopy, the responses reappeared.‡

In this paper we report isolation of a functionally active cilia preparation from olfactory rosettes of rainbow trout. Because no single biochemical criterion unique to cilia is available, the preparation was characterized by using several biochemical markers known to be associated with cilia; also, the cilia were observed by electron microscopy. Functional activity was measured by binding of ^3H-labeled odorant amino acids. A preliminary report of this work has appeared.‡

MATERIALS AND METHODS

Materials. Rainbow trout were decapitated and the heads were transported to the laboratory on ice. Unlike our previous studies in which frozen tissue was used (4), the present experiments were carried out on fresh tissue.

Reagents and their suppliers were as follows: L-[2,3-^3H]alanine (16 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), L-[*G*-^3H]-serine (2.8 Ci/mmol), D-[1-^{14}C]alanine (17.9 mCi/mmol), and L-[*G*-^3H]threonine (2 Ci/mmol) from New England Nuclear; L-[4,5-^3H]lysine (40 Ci/mmol) and D-[*U*-^{14}C]alanine (43 Ci/mmol) from Amersham/Searle; unlabeled amino acids, Tris base, *N*-(2-acetamido)-2-aminoethanesulfonic acid (Aces), NADH, phospho*enol*pyruvate (monopotassium salt), ATP (disodium salt), ouabain, pyruvate kinase from rabbit muscle (EC 2.7.1.40, type III), L-lactate dehydrogenase from rabbit muscle (EC 1.1.1.27, type XI), bovine serum albumin, adenine, guanine, and EDTA (disodium salt) from Sigma; Coomassie brilliant blue R and bromphenol blue from Fisher; Millipore filters (type HAWP, 0.45-μm pore size) from Millipore; and thin-layer chromatography plates (20 × 20 cm) coated with cellulose (MN 300, 250 μm thick), from Analtech (Newark, DE). Other chemicals were reagent grade.

Isolation of Cilia. The method used was based on the ethanol/calcium procedure of Watson and Hopkins (10) with the modifications of Linck (11) and additional modifications as noted below. In a typical preparation, a batch of about 100 rosettes (4 g, wet weight) was dissected onto a cold glass plate. In some experiments, up to eight batches were used. Further steps were carried out at 0–4°C. Unlike previously (4), the rosettes were not trimmed of excess tissue, in order to minimize processing time and possible damage to the cilia. Each batch of rosettes was placed into 30 ml of 10% (vol/vol) ethanol/0.1 M NaCl/2 mM EDTA/30 mM Tris·HCl, pH 8.0. After the sample was gently stirred for 2 min, it was brought to 10 mM in $CaCl_2$ and gently stirred for an additional 18 min. The

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Abbreviation: Aces, *N*-(2-acetamido)-2-aminoethanesulfonic acid.

* This is paper no. 2 in a series. Paper no. 1 is ref. 4.

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‡ Rhein, L. D. & Cagan, R. H. (1979) Abstracts of the 11th International Congress of Biochemistry, Toronto, Ont., July 8–13, 1979, p. 576.

mixture was centrifuged at 1500 × *g* for 5 min on a Sorvall RC2-B centrifuge. The resulting pellet contained the deciliated rosettes. The supernatant, which contained the cilia, was carefully decanted through two layers of gauze and then centrifuged at 10,000 × *g* for 10 min. The cilia pellet was washed twice by suspending it in 10 mM Tris·HCl buffer (pH 8.0) and centrifuging at 10,000 × *g* for 15 min. The supernatants were decanted and discarded. For binding experiments, the buffer for the two wash steps was 50 mM Aces at pH 7.0 (4) rather than Tris. The washed cilia pellet was then suspended for binding or other assays.

Preparation of Rosette Homogenate. Whole homogenates were prepared (0–4°C) from 50 rosettes in a typical preparation. Based on the wet weight, a 15% homogenate in 10 mM Tris·HCl (pH 8.0) was prepared by manual homogenization (25–30 strokes) in an all-glass TenBroeck homogenizer with a tight-fitting pestle. The sample was diluted with the Tris buffer for chemical and enzyme analyses. Deciliated rosettes obtained from the pellet after the initial centrifugation (see above) were treated identically to the whole rosettes.

Analyses of Adenine and Guanine. The approach was similar to that of Stevens *et al.* (12). To 1.4-ml samples in 10 mM Tris·HCl (pH 8.0) was added 0.2 ml of 80% (wt/vol) trichloroacetic acid. A buffer blank without tissue was routinely carried through the procedure. The mixture was maintained on ice for 30 min with periodic stirring (12) and then centrifuged. The supernatant was extracted twice with 2 vol (each time) of ether saturated with water. The water phase was evaporated to dryness under N_2. Each sample was heated for 1 hr at 125°C in a screw-cap test tube with 0.5 ml of 88% formic acid (13). After evaporation to dryness, the sample was taken up in 1.2 M HCl and applied to the corner of a thin-layer plate that, prior to use, had been washed twice with each of the developing solvents and air-dried after each wash.

The plate was developed in two dimensions. The first solvent (14) was *n*-propanol/2 M HCl, 65:35 (vol/vol), and the second solvent (15) was *n*-butanol/methanol/water/concentrated ammonium hydroxide, 60:20:20:1 (vol/vol). Guanine and adenine spots were detected under ultraviolet light and identified by comparison with standards. The appropriate areas were scraped into test tubes and the adsorbent was extracted with three 1-ml portions of 88% formic acid. After evaporation to dryness, the extracts were dissolved in 1.2 M HCl and quantified spectrophotometrically at 260 nm on a Zeiss PMQII spectrophotometer. Recoveries of adenine and guanine were similar (62–68%) as determined with authentic nucleotides; because ratios are reported, the values are not corrected for losses.

ATPase Assay. Activity of ATPase (ATP phosphohydrolase, EC 3.6.1.3) was measured by using the method of Pullman *et al.* (16) with several modifications (11, 17). The initial 1-ml reaction mixture contained 50 mM Tris·HCl at pH 8.0, 3 mM $MgCl_2$, 0.2 mM NADH, 2 mM phospho*enol*pyruvate, 1 mM ouabain, 40 units (60 μg) of pyruvate kinase, and 40 units (50 μg) of lactate dehydrogenase. After addition of substrate (2 mM ATP), the decrease in absorbance at 340 nm (25°C) was recorded continuously for 15 min in a Cary model 14 recording spectrophotometer. Each sample was assayed at least in duplicate and at two protein concentrations. Units of enzyme activity were calculated with $\varepsilon = 6.22 \times 10^{-3}$ M^{-1} cm^{-1} for NADH, which is related stoichiometrically to the P_i produced.

Disc Gel Electrophoresis. Samples were analyzed by discontinuous polyacrylamide gel electrophoresis on 11-cm gels in the presence of sodium dodecyl sulfate (18) with some modifications. Kirschner *et al.* (19) used this procedure to separate α- and β-tubulins. The gels contained 10% rather than 7% acrylamide, the sample was heated for 5 min, and electrophoresis was carried out at 37°C to reduce the running time to 2.5 hr. About 25–30 μg (protein) was applied to each gel. The gels were stained with Coomassie brilliant blue R and destained (20). A tubulin standard from chicken embryo brain was a gift from Joseph Bryan. The stained gels were scanned at 560 nm by using a Zeiss PMQII spectrophotometer equipped with a linear transport accessory (Vicon, model 1050), a transmittance-extinction (TE) converter, and a Leeds and Northrup strip-chart recorder (Speedomax, XL680).

Electron Microscopy. The cilia pellet was fixed in buffered 3% glutaraldehyde (pH 7.0) and postfixed in 1% osmium tetroxide. It was briefly washed, dehydrated with ethanol, and then stained with 4% uranyl acetate in 75% ethanol. The samples were infiltrated with propylene oxide/Epon 812 mixtures and embedded in Epon 812. Thin sections were stained with 8% uranyl acetate in 50% ethanol and with lead citrate and were examined by using a Hitachi HU-11C-1 transmission electron microscope.

Odorant Binding Assay. Binding of amino acids (4, 21) is operationally defined and was measured as described (4) but with the assay volume decreased by one-half. Parallel samples containing excess unlabeled amino acid were used to measure nonspecific entrapment. The sample was incubated on ice for 1 hr, replicate 0.5-ml aliquots were filtered and rinsed, and the radioactivity was determined as described (4). Saturation curves were determined over a ligand concentration range of 10^{-7} to 10^{-4} M. Protein concentration was between 20 and 120 μg/ml in the assay mixture and was quantified (22) with bovine serum albumin as a standard.

RESULTS

Isolation of Cilia. A procedure to remove cilia selectively from mollusc gills was reported by Linck (11). By using that procedure, we isolated, from whole olfactory rosettes of the rainbow trout, a preparation enriched in ciliary material. Overall, the recovery of protein was quantitative, with only 0.5% of the whole rosette protein appearing in the cilia preparation. The procedure yielded about 30 μg of protein per fish or 0.35 mg per g (wet weight) of rosettes. [From mollusc gills, Linck (11) obtained 1.2 mg of protein per g (wet weight) of gills. Because excess tissue was not removed during dissection, our yield of cilia was lower per wet weight of tissue.]

Previous studies (4) of odorant binding activity with a different type of sedimentable fraction (fraction P2) prepared from homogenates of trout olfactory rosettes showed that binding activity was stable when the tissue had been stored at −65°C for 2 weeks. In preliminary experiments with cilia preparations, rosettes were dissected from frozen-thawed trout heads and washed to remove mucus (4) prior to deciliation. The yield of cilia was lower, averaging 3–4 μg of protein per fish but with levels of specific binding activity similar to those reported here. Frozen storage of the trout heads and washing the rosettes prior to deciliation apparently caused disruption of cilia. Accordingly, these steps were eliminated, thereby increasing the yield of the cilia preparation by about 8- to 10-fold.

Biochemical Markers of Cilia. To assess the enrichment of ciliary material, the preparation was examined for an enzymatic marker and two chemical markers; electron microscopy was used to ascertain the presence of cilia morphologically. A Mg^{2+}-activated ATPase is the major constituent of the cilia dynein arms (23). Dynein arms are present in axonemes of cilia in the olfactory region of trout (unpublished data; see Fig. 2*E*). The specific activity of Mg^{2+}-ATPase in the cilia preparation was enriched about 4-fold compared with that in the whole rosette homogenate (Table 1). Although not conclusive by itself,

Table 1. Activity of Mg^{2+}-ATPase in cilia preparation from trout olfactory rosettes

Sample	Mg^{2+}-ATPase Total activity,* μmol/min	Mg^{2+}-ATPase Specific activity, nmol/min per 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

* The values for total activity are expressed per g 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 from 300 fish.

this result supports the hypothesis that the preparation is enriched in cilia.

Noncovalently bound GTP is associated with microtubules (24). The outer doublets of the axonemal microtubules of cilia and flagella have associated with them guanine derivatives and essentially no adenine derivatives (12, 24). The ratio of the noncovalently bound guanine and adenine derivatives therefore was chosen as a criterion to demonstrate enrichment of cilia. The ratio of guanine to adenine (Table 2) of 1.73 for the cilia is 2.6-fold higher than that in the whole rosettes and 22-fold higher than that in the deciliated rosettes. The selective loss of guanine derivatives from the whole rosettes indicates the loss of cilia during deciliation. These results support the hypothesis that the preparation is enriched in ciliary material.

As the major structural protein of microtubules, tubulin (23, 24) was chosen as a marker for cilia. The gel electrophoresis conditions used (18) are known (19) to separate tubulin into its subunits. The protein pattern in the cilia preparation was clearly different from that in whole rosettes, with fewer protein bands present in the cilia preparation (Fig. 1). Bands were observed at the same migration distances as the tubulin standard; these bands were present in larger amounts relative to the total protein in the cilia than in the whole rosettes. These findings also support the hypothesis that the preparation is enriched in ciliary material.

Electron Microscopic Examination. Transmission electron microscopy revealed cilia in the preparation (Fig. 2 *A–C*), along with substantial amounts of membranous and some microtubular material. Dynein arms were observed on the outer doublets. Although the numerous membrane profiles observed in the preparation could be fragments of nonciliary membranes, it is important to note that trout olfactory cilia are unusual in having large portions of the outer cilia membrane expanded into vesicular structures (Fig. 2 *D* and *E*). The membrane material therefore might also originate from the cilia. Occasional mitochondria, microsomes, and rough endoplasmic reticulum were observed, suggesting some cell damage during deciliation. Electron microscopy therefore reveals the presence of cilia although it is not suitable for quantitation.

Table 2. Nucleotide base content of the cilia preparation from trout olfactory rosettes

Sample	Nucleotide base content* μg/g wet weight Guanine	Adenine	μg/mg protein Guanine	Adenine	Ratio†
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 wet weight is that of the original whole rosette sample. Protein refers to the particular fraction. The results are from a single cilia preparation from 385 fish (770 rosettes).

† Guanine/adenine.

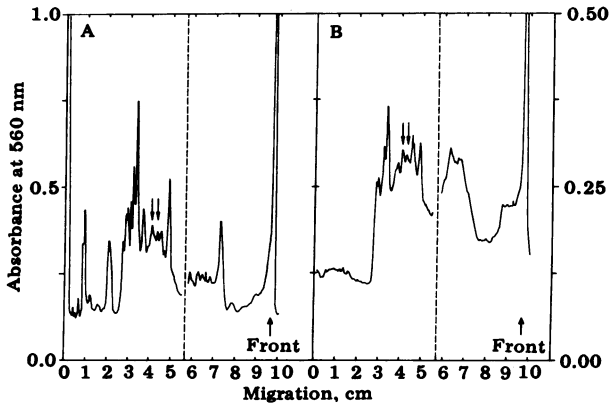

FIG. 1. Gel electrophoretic patterns of proteins from whole rosette homogenate (*A*) and cilia (*B*). The arrows indicate the migration of α- and β-tubulin bands of the standard. Patterns from triplicate samples were similar. The broken vertical line designates where the gel was sectioned to be accomodated by the scanning unit. Migration distance is measured from the top of the gel.

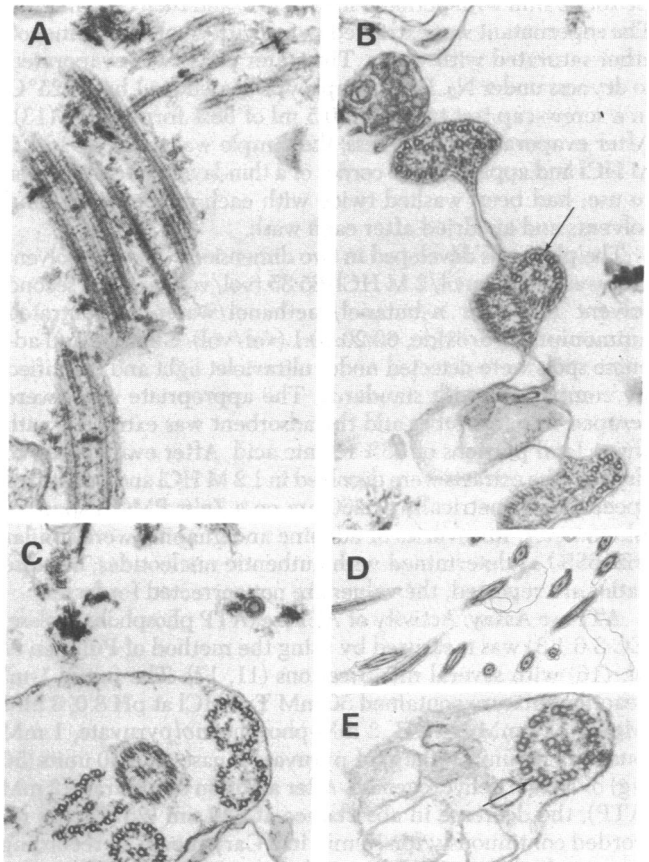

FIG. 2. (*A–C*) Transmission electron micrographs of the cilia preparation from trout olfactory rosettes. Arrows in *B* and *C* denote dynein arms. (*A*, ×37,100; *B*, ×32,300; *C*, ×32,300.) (*D*) Transmission electron micrograph of cilia *in situ* in the trout olfactory rosette. (×5,500.) (*E*) High power view of a trout cilium *in situ*, showing the dynein arms (arrow) and the expanded membrane. (×50,800.) The electron micrographs indicate the ultrastructural features of the cilia present but are not representative of the morphological composition of the total field which also contained large amounts of membranous, vesicular, and some microtubular material.

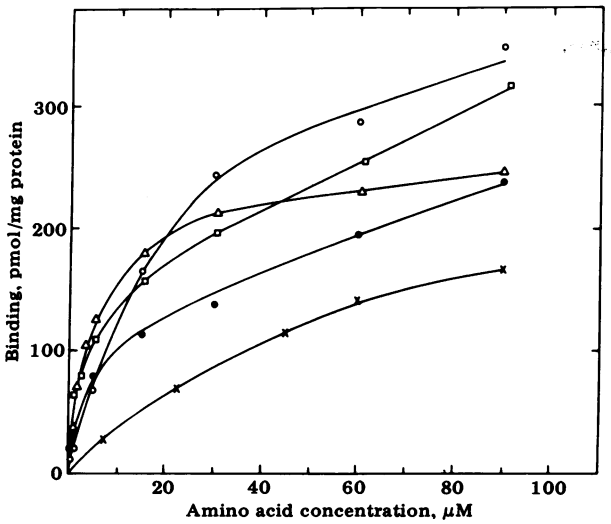

FIG. 3. Binding of odorant amino acids to cilia from rainbow trout olfactory rosettes. The data for each amino acid are the mean values of five preparations for L-alanine (●) and L-serine (△), three preparations for L-threonine (□), two preparations for D-alanine (X), and one preparation for L-lysine (○). The binding value of 252 pmol/mg for D-alanine at 180 μM is omitted in the interests of space.

Binding of Odorant Amino Acids. Binding measures an initial event in odorant recognition in the rainbow trout. Cagan and Zeiger (4) demonstrated that the relative order of binding of 10 odorant amino acids to a sedimentable preparation (fraction P2) compared closely with their reported (3) relative electrophysiological effectiveness as olfactory stimuli. Based on the biochemical studies (4), several types of binding sites were postulated, including site TSA which binds L-threonine, L-serine, and L-alanine, site L which binds L-lysine, and site A_D which binds D-alanine. Binding properties of the trout cilia preparation were therefore examined in order to verify the biological activity with respect to odorant recognition.

Binding curves (Fig. 3) and Scatchard analyses (25) were used to estimate the apparent dissociation constant (K_d) and maximal binding (B_{max}) for each amino acid (Table 3). The Scatchard plots for L-threonine, L-alanine, and L-lysine indicated the presence of two types of binding sites of different affinities. Those for L-serine and D-alanine indicated only a single affinity site for each. The results with the cilia preparation thus confirm the earlier findings in our laboratory with fraction P2 (4).

The data shown in Fig. 3 and Table 3 establish the activity of the isolated cilia preparation, although some details differ from the results with fraction P2 (4). Additional studies will be required to establish the specificities of binding sites TSA, L, and A_D to which these amino acids correspond. Preliminary results of competition studies with the cilia preparation using L-[^{3}H]alanine (5 μM) as the ligand and unlabeled L-serine, L-threonine, or L-lysine (each at 60 μM) as the competing amino acid revealed that L-serine and L-threonine compete with L-[^{3}H]alanine for binding but that L-lysine does not compete. These results confirm the previous findings (4) with fraction P2.

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

	High affinity		Low affinity	
Amino acid	K_d, μM	B_{max}, pmol/mg	K_d, μM	B_{max}, pmol/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 results shown are averages of those calculated from Scatchard plots of each individual preparation; the binding curves are shown in Fig. 3. n, Number of preparations analyzed.

To ascertain whether the binding of amino acids to the cilia may include active transport into vesicles, binding of L-[^{3}H]-alanine (5 μM) was assayed in the presence of 10 mM NaCl, because vertebrate amino acid transport systems are primarily dependent on Na^+ (26). No difference in binding of L-[^{3}H]-alanine in the presence (69 pmol/mg) or absence (64 pmol/mg) of Na^+ was observed.

DISCUSSION

The role of cilia in olfaction is controversial. For many years cilia were postulated (5, 6) to be involved in the initial phase of olfaction. A preliminary report by Tucker (7) has been widely cited as indicating that they are not functional in odorant recognition. He stated that removing turtle olfactory cilia with detergents did not affect electroolfactogram and neural responses. Moulton (27) argued against an essential role of cilia in olfaction. In a recent study by Bronshtein and Minor (9), brief exposure of frog olfactory epithelium to 0.1–0.15% Triton X-100 resulted in destruction of cilia and decline of the electroolfactogram response. Electron microscopy revealed that the cilia had been removed and also confirmed their regeneration within 2–3 days, at which time the electroolfactogram responses recovered. This latter study therefore supports a role of cilia in the olfactory response.

Elucidation of the role of cilia in odorant recognition and examination of the specificity and mechanisms of odorant–cilia interactions are difficult to achieve without obtaining a functionally active, isolated cilia preparation. Previous studies (4) showed the importance of the initial binding interaction in recognition of odorants. An approach was therefore designed utilizing the binding assay as a measure of functional activity, and a previous approach (10, 11) to isolate cilia was adapted to the present system.

No single criterion is definitive in establishing the presence and degree of enrichment of ciliary material in an isolated preparation. Several criteria were therefore selected based on constituents known to be associated with cilia of other cells and tissues. Direct visualization of cilia was accomplished by electron microscopy, which reveals their presence but is not quantitative.

In some cases olfactory cilia have been reported to lack dynein arms (28, 29) but dynein arms are present in the cilia in the olfactory region of trout (Fig. 2). Dynein arms contain Mg^{2+}-ATPase activity (23) which is essential for motility. It has been shown (5, 30) that olfactory cilia are motile, including a recent preliminary report (31) that the motility of cilia in the frog olfactory epithelium increased and became synchronized in the presence of chemical stimuli. As the major constituent of dynein arms, the Mg^{2+}-ATPase serves as an appropriate marker for cilia although it also is present in other cellular structures (e.g., ref. 16). A significant increase in its specific activity was observed in the cilia preparation (Table 1). The specific activity in the trout homogenate (74 nmol/min per mg of protein) is similar to that reported in homogenates of dog olfactory epithelium (17). Compared with other isolated cilia preparations, however, the present enzyme had relatively higher activity (275 nmol/min per mg of protein). For example, Linck (11) reported 100 nmol/min per mg of protein for the mollusc gill cilia.

The content of guanine nucleotides in the cilia preparation was substantially enriched in relation to the adenine nucleotides; the ratio was 22-fold higher than with the deciliated rosettes (Table 2). Microtubules contain 2 mol of bound guanine nucleotide per tubulin dimer (24), and the outer doublets of cilia contain associated guanine derivatives but not adenine derivatives (12). The guanine content in the olfactory cilia preparation was comparable with that of other cilia—for example, 1 mol of guanine derivatives per 10^5 g of whole cilia protein from *Tetrahymena* (12) compared with 1.3 μg of guanine per mg of cilia protein (Table 2) or 0.9 mol of guanine per 10^5 g of cilia protein from the trout olfactory rosette.

Tubulin is the major structural protein of microtubules and its two subunits, α-tubulin and β-tubulin, are separable by disc gel electrophoresis (19). Although such identification is not conclusive, the evidence (Fig. 1) is consistent with the presence of tubulin and suggests its enrichment in the cilia preparation.

All the biochemical markers for cilia were therefore present in the preparation. Although any one criterion by itself may not provide compelling evidence, the presence of several criteria strongly supports the conclusion of substantial enrichment in ciliary material, and the identification of cilia by electron microscopy (Fig. 2 *A–C*) provides corroborative evidence. Functional activity of the cilia preparation in relation to odorant recognition was assessed by its binding activity for five odorant amino acids (4). The values of apparent K_d estimated by Scatchard analyses (25) (Table 3) confirm those determined with the same amino acids for the sedimentable fraction (fraction P2) isolated previously (4); B_{max} appears to be reduced due to denaturation of receptors by the ethanol in the deciliation medium. Preliminary competition studies indicated the presence of separate sites (TSA and L) in the cilia preparation. It is concluded that olfactory receptors are active in the isolated cilia preparation.

The present approach provides a clearer definition of the role of cilia in olfaction. Isolation from the olfactory epithelium of a cilia preparation with odorant binding activity provides biochemical support for the hypothesis that receptor sites for odorant molecules are part of the cilia. We suggest that the receptor molecules, which are probably proteins, are an integral part of the cilia membrane. More detailed localization of the receptor sites along the cilia membrane and the nature of the olfactory receptor macromolecules remain questions for future studies.

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