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STUDIES WITH A PROTEIN PURIFIED FROM RAT OLFACTORY BULBS WHICH IS SPECIFIC TO THE OLFACTORY CHEMORECEPTOR NEURON

by

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SUMMARY

The olfactory marker protein was isolated from rat olfactory bulbs and characterized with regard to its immunological and physicochemical properties.

Antisera against the rat olfactory marker protein were prepared by injection of the homogeneous protein into a goat and a rabbit. When the antisera were tested by immunodiffusion against olfactory tissue extracts, many, but not all, mammalian species cross-reacted against these antisera. Immunoprecipitin titrations with the goat antiserum generally showed higher cross-reactivity against olfactory extracts from species more closely related to the rat. Human olfactory bulb extracts and non mammalian olfactory tissue extracts did not cross-react with the antisera by either immunodiffusion tests or immunoprecipitin titrations. However, they did cross-react when tested by a competitive binding radioimmunoassay using tritium labeled purified rat protein and the goat antibody.

The olfactory marker protein,which is an example of a brain protein specific to one cell, the olfactory chemoreceptor neuron, has a very wide species distribution being present in rat, mouse, hamster, guinea pig, sheep, cow, rabbit, pig, dog, man, frog, and garfish. Therefore, it presumably plays an important and unique role related to the function of this primary chemosensory neuron.

The physicochemical properties of the rat olfactory marker protein were compared to those of the protein isolated from the mouse. The rat protein was less acidic (pI = 5.0) than the mouse protein (pI = 4.7). However, the amino acid compositions were very similar: in both proteins ARG + LYS accounted for 13 mole percent and GLU + ASP for 30 mole percent

of the total residues. Molecular weights of both proteins estimated by SDS gel electrophoresis were indistinguishable and estimated to be 16,500 daltons. The molecular weight of the native rat olfactory marker protein estimated by gel filtration techniques was 30,000 daltons which is identical to the molecular weight of the native mouse and garfish olfactory marker proteins. This suggested a dimeric structure. The purified rat and mouse proteins behaved like species of 35,000 daltons on gel filtration. During these studies, evidence was obtained that the rat olfactory marker protein undergoes conformational changes upon storage and during the purification procedure.

The evolutionary conservation of the olfactory marker protein has been strongly suggested by the immunological studies with the rat protein which showed species cross-reactivity with olfactory tissue extracts from a wide range of vertebrates. This has now been confirmed by direct analysis of the chemical and physical properties of the mouse and the rat proteins and by preliminary observations with the garfish olfactory marker protein.

The subcellular distribution of the olfactory marker protein was studied in rat olfactory bulbs. It was compared to the subcellular distribution of other biochemical markers. The highest relative specific activity of the olfactory marker protein was in the soluble compartment with 38% recovery. In contrast to the other soluble markers, a large amount of the olfactory marker protein, which became occluded during the homogenization in the 0.32 M sucrose, 1 mM $MgSO_4$ medium, was trapped in larger particles which sedimented at a lower speed. It is suggested that these particles are synaptic glomeruli fragments. The crude mitochon-

drial pellet, which contains most of the synaptosomes, was enriched in choline acetyltransferase.

The subcellular fractions were also characterized regarding their high affinity uptake properties. Preferential uptake was observed with choline and GABA in the crude mitochondrial pellet. No uptake was observed with carnosine in any fraction in those conditions.

Further enrichment in the large subcellular particles containing the olfactory marker protein was obtained on a continuous sucrose gradient.

The subcellular fractions enriched in the olfactory marker protein should be useful for *in vitro* studies concerning the function of this protein and the identification of the putative neurotransmitter at the primary olfactory synapse.

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GENERAL INTRODUCTION

The central nervous system is one of the most complex and fascinating tissues of any animal species. It is fascinating because of its function of controlling an animal's most complex behaviors such as learning, memory, and the ability to react to the environment and to modify it. The complexity of the structure of the nervous system seems unequaled. Two classes of very specialized cell types have been described, the glial cells and the neuronal cells, but in each of these classes, a large variety exists (1). This variety has been recognized both on morphological and on functional grounds. Very little is known about the biochemistry of glial cells (2). They have not been studied to the same extent as the neurons, having been considered as less important to the nervous functions. Recently, however, properties previously believed to be characteristic only of the neurons have been also described for glial cells (3). Moreover, direct functional relationships between glial and neuronal cells may exist (4). The extensive anatomical and physiological knowledge of the central nervous system has recently been utilized in attempts to develop models of neural organization (5). As a result, computer simulation of a cerebellum model has been conceived. This kind of work deals nearly exclusively with neuronal networks and largely ignores the presence of glial cells in the brain. However, it merely illustrates how complex the organization of the brain is and how little is understood concerning interactions among neurons within brain regions or between regions. Thus, our knowledge of neuronal physiology and

biochemistry mainly derives from the study of simpler systems such as the squid giant axon (6), or in the peripheral nervous system of vertebrates, the neuromuscular junction (7), the sympathetic ganglia (8), and the reflex arc (9). In those simpler cases, specific cells have been studied in great detail and provide a model for neurons of the central nervous system inaccessible to direct study.

The concept of chemical neurotransmission derived from experiments with the peripheral nervous system. T. R. Elliott, in 1904 (10), compared the effect of sympathetic nerve stimulation and of adrenaline on various tissues and suggested that epinephrine "might then be the chemical stimulant liberated on each occasion when the impulse arrives at the periphery". This idea was later tested by Otto Loewi (1921) in his classical experiment with the vagus innervation of the frog heart (11). It was later demonstrated that the substance released by the vagus nerve was acetylcholine. Increased understanding of neurotransmitter metabolism was further developed with the discovery at the neuromuscular junction of the quantal release of acetylcholine (12) and the observation with the electron microscope of the presence of synaptic vesicles in nerve terminals (13). Specific enzymes and proteins are associated with specific neurotransmitters. For example, the enzyme choline acetyltransferase is responsible for the synthesis of acetylcholine from choline and acetyl CoA (14) whereas the chromogranin proteins are released from the synaptic vesicles simultaneously with norepinephrine (15). Several compounds have now been proposed as putative neurotransmitters: amines such as acetylcholine, norepinephrine, dopamine, serotonin (16), histamine (17); amino acids such as γ-aminobutyric

acid (GABA) (18), glycine, glutamic acid (19), and recently, the peptide substance P (20). The available evidence indicates that one neuron is able to synthesize, store, and release only one neurotransmitter (21). Thus, classes of neurons are defined according to the transmitter that they contain. Moreover, the enzyme specific for the synthesis of a neurotransmitter is also characteristic for neurons of that class. Neurotransmitters and their synthetic enzymes constitute the first cell-specific genetic markers known in the nervous system. Those markers have been used extensively in studies of the central nervous system. Their levels have been modified during behavioral experiments (22) or after drug treatments (23), and monitored during subcellular fractionation (24) and in neuroblastoma cell cultures (25). Although electron microscope studies have shown that in the mammalian central nervous system most of the synapses are chemical synapses (26), known putative neurotransmitters can be attributed to only a few of those synapses (26, 16). Thus, the neurotransmitters at most of the central synapses are essentially unidentified.

A search for nervous system specific protein markers was undertaken by Moore in 1965 (27) and resulted in his isolation of the glial specific protein S100 and of the neuronal specific protein 14-3-2 (28). These proteins were uniquely located in nervous tissue and had a bread species distribution (29). It was, therefore, suggested that the presence of specific proteins in the glial or neuronal cells were necessary for their specific functions. Following this work, a new field developed and many nervous system specific proteins have now been isolated (58-64). In a slightly different approach, Margolis was searching for

protein markers of individual cell types. The underlying principle was that cell specific genetic markers should allow the study of individual cell types in the midst of various other cells without disrupting the whole structure as is often done in biochemical studies of the central nervous system. A protein specific to the mouse olfactory bulbs was isolated (30). The choice of the mouse as the experimental animal was motivated by the fact that this is the mammalian group in which the most genetic variants are known (31). The olfactory bulb represents a brain region which presents interest from several view points. Olfaction has been implicated in modulating important behaviors such as those related to reproduction (32), territoriality (33), and food searching (34). The olfactory bulbs are the site of the primary synapse from the olfactory chemoreceptor neurons (35). Biochemical studies with a specific marker to these olfactory bulbs may thus allow the understanding of the behavioral and biochemical events which follow olfactory sensory inputs.

The olfactory chemoreceptor neurons constitute the primary olfactory pathway (Fig. 1). Their cell bodies are present in the nasal olfactory mucosa which contains non neuronal cells as well. The unmyelinated axons of the olfactory chemoreceptor neurons, which form the first cranial nerve or olfactory nerve, extend to the olfactory bulbs where they terminate in a layer of rounded structures, the synaptic glomeruli. The olfactory fibers do not divide before entering the glomeruli (although they may branch inside) where they form synapses with the primary dendrites of the mitral and tufted cells. Therefore, the axon of any receptor cell does not terminate in more than one

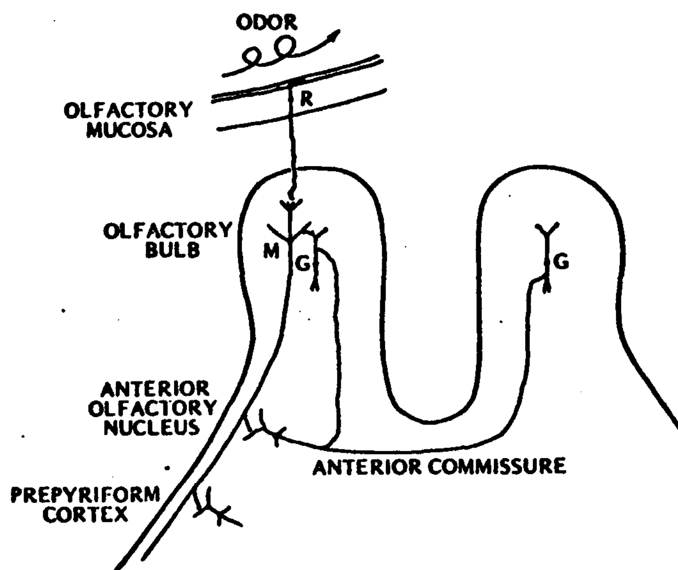


Fig. 1. Connections of the olfactory bulb. R, olfactory receptor cell; M, mitral cell; G, granule cell. From Shepherd (35).

glomerulus and thus, each glomerulus receives impulses from a distinct receptive field. Allison and Warwick (1949) (36) estimated that in the rabbit every glomerulus receives impulses from " an average of 26,000 receptors and passes impulses onto 24 mitral cells and 68 tufted cells". This illustrates the way input is condensed and suggests that the glomeruli serve as processing centers. The histologists of the late nineteenth century studied the laminated structure of the olfactory bulbs in great detail. It appears that the olfactory bulb structure is very similar in all vertebrate species (37). More recently, anatomical studies, at the light microscope level as well as at the electron microscope level (38), (39) combined with electrophysiological studies (40), have permitted the analysis of the structure of the olfactory bulbs and the interaction between cells of different layers. As a result of these studies, the olfactory bulb has been proposed as a model cortical system (41). Although it is structurally similar to other cortical regions, it is one of the first cortical structures to have emerged in the course of vertebrate evolution and, despite its complexity, it is one of the simplest in construction.

The peculiar anatomy of the olfactory bulbs presents advantages which have been used in electrophysiological, anatomical, and biochemical studies (35). The olfactory bulbs of laboratory animals such as mice, rats, and rabbits are situated in front of the brain and thus easily exposed for the purpose of recording with microelectrodes and applying neurally active agents by micropipettes. The main input to the bulbs is by the bundles of olfactory nerve fibers coming from the nasal olfactory epithelium. The main output is by the lateral olfactory tract

bundles on the surface of the prepyriform cortex of the brain. In electrophysiological studies, one stimulating electrode may be placed on the lateral olfactory tract to stimulate the olfactory bulb by invading the mitral cells antidromically. Another electrode may be placed on a bundle of olfactory nerve fibers so that orthodromic stimulation can be applied and cause activation of the mitral cells through the mitral cell's primary dendrites. The bulb can thus be activated by input and output pathways that are completely separated from each other (40). In anatomical studies, degeneration experiments have been performed after lesion of the olfactory epithelium (38) section of the olfactory nerve (42) or of the lateral olfactory tract (43). The structure of the olfactory bulbs, after those lesions were analyzed at the electron microscope level, led to a better understanding of the connections between cells of different layers. Bulbectomy resulted in degeneration of the olfactory chemoreceptor neurons in the olfactory epithelium (38). There is accumulating evidence that regeneration of these neurons may occur (44). This approach was used by Margolis to study the location of biochemical markers in this tissue. The mouse olfactory marker protein had been isolated from the olfactory bulbs (30). A further study (45) demonstrated that this protein was not synthesized in the olfactory bulbs but in the olfactory epithelium, where it was also present in high concentrations . This finding suggested that this protein was uniquely localized in the primary olfactory chemoreceptor neurons. Disappearance of the olfactory marker protein in the epithelium following bulbectomy and in the olfactory bulbs following destruction of the epithelium by zinc sulfate irrigation confirmed this hypothesis (46).

Recently, these same degeneration procedures were used in a search for the putative neurotransmitter involved at the primary synapse between the olfactory nerve and the mitral cells (47). Although none of the known putative neurotransmitters appeared to be involved in this pathway, the dipeptide carnosine [β-alanyl-L-histidine] was shown to behave like the olfactory marker protein in its response to degeneration procedures. Thus, carnosine appears to be also a highly specific marker for the olfactory chemoreceptor neuron. However, in contrast to the olfactory marker protein, carnosine is also present in high concentrations in striated muscle tissue. The synthetic enzyme carnosine synthetase also behaves as a highly specific marker of the olfactory chemoreceptor neuron, whereas the levels of the degradative enzyme carnosinase does not. Thus, carnosine synthetase, but not carnosinase decreases following zinc sulfate irrigation or bulbectomy. Carnosinase, therefore, is not a specific marker for that pathway (48).

My main objectives during this work have been:

1. to demonstrate that the olfactory marker protein, first isolated from the mouse olfactory bulbs, indeed was a specific marker of broad species distribution. I, therefore, isolated the olfactory marker protein from rat olfactory bulbs and characterized it with regard to its immunological properties (Chapter I) and its physicochemical properties (Chapter II).

2. to approach the question of the function of the olfactory marker protein. I decided to study the subcellular distribution of the rat marker protein in the olfactory bulbs, to compare it to the distribution of other biochemical markers, and to attempt the isolation of

subcellular particles enriched in the olfactory marker protein for use in further *in vitro* studies of synaptic function (Chapter III).

(I) IMMUNOLOGICAL STUDIES OF THE RAT OLFACTORY MARKER PROTEIN

The olfactory marker protein had been isolated from the mouse olfactory bulbs and preliminary physicochemical characterization carried out (30). A study of the site of biosynthesis of the mouse olfactory marker protein indicated that it is synthesized in the olfactory mucosa, presumably by the olfactory chemoreceptor neurons, and is probably transported by axoplasmic flow directly to the synaptic terminals of these neurons in the olfactory bulbs (45, 46). This protein is restricted to the primary olfactory pathway and, therefore, is presumed to play an important and unique role related to the specific function of these primary chemosensory neurons.

In previous studies, an antiserum prepared in goat against the purified mouse olfactory marker protein, cross-reacted immunologically only with extracts of the olfactory bulbs and epithelia of rodents, i.e. rat, mouse, hamster (30). This observation could indicate either a very restricted specificity of the antiserum or a very restricted species distribution of the protein. In order to resolve this apparent ambiguity, the protein was isolated from another species and antisera were prepared against that olfactory protein. For these studies, I chose the rat because, although its olfactory marker protein cross-reacted with the antibody to the mouse olfactory protein, preliminary studies had indicated that it manifests physical and chemical differences from the mouse protein.

MATERIALS AND METHODS

Materials. The polystyrene beads were purchased from the Dow Chemical Company, USA as a 10% aqueous suspension and NaB^3H_4 (8.4 Ci/mmol) from Amersham/Searle. Fluorescamine was furnished by Roche Diagnostics, Nutley, New Jersey. All other materials were of analytical reagent grade.

Rodents, guinea pigs, rabbits, and chickens were obtained from local breeders and were killed by CO_2 asphyxiation followed by exsanguination. Sheep, cow, pig, and goat heads were purchased from local slaughter houses, packed in ice, and dissected on arrival in the laboratory. Northern grass frogs were purchased from Nasco, Fort Atkinson, Wisconsin. Frozen garfish bills were obtained from Gulf Specimen Company, Inc., Panacea, Florida. Frozen human olfactory bulbs were kindly supplied by Dr. M. Lyons from autopsy material.

The rat olfactory marker protein was isolated following a method very similar to that described for the mouse olfactory protein (30). This method is reported in detail in Chapter II. Initially, the preparation was monitored by taking advantage of the cross-reactivity of the heterologous rat olfactory bulb protein in the homologous mouse olfactory protein radioimmunoassay (49). Subsequently, a homologous rat olfactory protein radioimmunoassay was utilized as described below.

The partially pure protein (about 600 μg) was separated from any trace of contaminants by electrophoresis on 14% acrylamide gels (Chapter II). After being made visible by fluorescent staining with 1-anilino-naphthalene-8-sulfonate under non denaturing conditions (50),

the pure protein bands in polyacrylamide gels were then excised. The pooled slices were homogenized in 5 ml of 0.15 M NaCl, 10^{-3} M EDTA acid, adjusted to pH 7.5 with Tris (EDTA-Tris pH 7.5) and emulsified with an equal volume of Freund's complete adjuvant. A 500 μg portion of the protein was injected subcutaneously into a goat, and the remaining 100 μg were injected intradermally into a rabbit. Blood was then collected weekly (about 20 ml from the goat and about 1 ml from the rabbit), allowed to clot, centrifuged at 800 g for 30 min, and the sera collected. Antibody formation was monitored by Ouchterlony double immunodiffusion tests on agarose plates (Cordis Labs). After three months, a booster injection of similarly prepared protein was administered (400 μg to the goat, 100 μg to the rabbit). Blood was collected and tested twice weekly for three weeks when a large amount of blood was collected. The antisera were stored frozen at -8°C in small aliquots.

Soluble tissue extracts were prepared by homogenization of the olfactory tissues with 3 to 10 volumes of 0.01 M NaH_2PO_4/Na_2HPO_4 buffer, pH 7.7 followed by centrifugation at 100,000 g for 1 hour. Quantitative immunoprecipitin reactions were performed following the procedure described by Kabat and Mayer (51) using sera which had also been centrifuged for 1 hour at 100,000 g. To 3 ml conical tubes were added 100 μl of antiserum, preimmune serum, or water followed by 100 μl of Tris HCl 0.06 M, pH 7.4 in 0.90 M NaCl, variable amounts of extract and distilled water to a final volume of 0.6 ml. The tubes were incubated for 1 hour at 37°C and then kept at 4°C for 2 days. The resulting immunoprecipitates were washed 3 times by repeated centrifugation at 800 g for 30 min and resuspension with 2 ml of 0.01 M NaH_2PO_4/Na_2HPO_4 buffer, pH 7.4

containing 0.15 M NaCl. The washed precipitates were dissolved in 100 μl of a 0.1 M NaOH containing 2% Na_2CO_3 and 0.1% sodium dodecyl sulfate. Each immunoprecipitin reaction was performed in duplicate and the protein content routinely assayed by the fluorescamine method (52) with bovine serum albumin as a standard, except in the time course study where we used the method of Lowry (53). In order to use the fluorescamine assay, which is about 10 times more sensitive than the method of Lowry and much more rapid, it is essential to avoid using any buffer containing primary amines when washing the immunoprecipitates. To each 100 μl of protein solution, 1.6 ml of 0.05 M $NaH_2PO_4/NaHPO_4$ buffer, pH 8.0 was added, followed by 0.5 ml of fluorescamine (30 mg/100 ml) in dioxane, and immediate mixing. The protein content of the crude tissue extracts was assayed with fluorescamine after filtration on Sephadex G-25 columns, 20 x 0.7 cm, eliminating all the low molecular weight amines (54).

A solid phase radioimmunoassay was developed using polystyrene beads of 0.5 μ diameter (55) as follows: one ml of the goat antiserum was diluted to 100 ml in 0.1 M $NaHCO_3/Na_2CO_3$ buffer, pH 9.6; 10 ml of a 10% suspension of polystyrene beads was added to the diluted antibody solution and the mixture allowed to stand at room temperature overnight. In order to remove any loosely bound antibody, the beads were then centrifuged for 10 min at 27,000 g and washed twice by resuspension in 0.15 M NaCl containing 10^{-2} M Tris-HCl and 0.1% sodium azide buffer, pH 7.4 in the presence of 0.1% bovine serum albumin. The purified rat protein was tritiated by reductive alkylation with tritiated sodium borohydride and formaldehyde at pH 9.0, as described previously (49). Purified rat

olfactory marker protein was used as a standard. The assay was performed in 10 x 75 mm disposable glass tubes (Bellco). Antibody coated beads (0.3 ml) were added to 0.4 ml of labeled rat protein (about 8000 cpm), 0-0.3 ml tissue extracts and enough 0.15 M NaCl, 10^{-2} M Tris-HCl, 0.1% sodium azide buffer pH 7.4 to give a final volume of 1 ml. After mixing, the tubes were incubated for at least 1 hour at 37°C, centrifuged for 10 min at 27,000 g, and the supernatants removed by aspiration. The beads were washed once by suspension and recentrifugation with the same buffered saline as used in the reaction. The radioactivity bound to the beads was then measured after transferring the pellet to scintillation vials with three 1 ml portions of H_2O and adding 10 ml of Aquasol (New England Nuclear). The sensitivity of the method could be manipulated by altering the incubation time beyond 1 hour (55) to as much as 16 hours. Thus, a standard curve was routinely constructed with each set of assays.

RESULTS

Immunodiffusion tests. A positive and specific reaction was observed on Ouchterlony plates with boosted sera from both goat and rabbit against extracts of olfactory bulbs and olfactory epithelia from all the tested mammalian species, except those from the goat and human (Table 1). A single sharp immunoprecipitin band of identity appeared against both antisera. In contrast, when extracts of cerebellum or other parts of the brain were used, no cross-reaction was visible. A typical example is presented in Figure 1 in which rabbit antiserum was reacted with extracts of olfactory bulb or epithelium from several species as well as mouse cerebellum. Except for the goat extract, all the other tissue extracts formed precipitin bands of identity but of variable intensity while no precipitate was observed with the mouse cerebellar extract. No precipitate was observed when the antiserum was replaced by preimmune serum. No cross reaction was seen by immunodiffusion with olfactory tissue extracts from the non-mammalian species tested, i.e., chicken, frog, garfish (Table 1). Essentially, identical results were obtained with the goat antiserum.

Quantitative immunoprecipitin tests. A study of antibody response in the goat was carried out by quantitative immunoprecipitin titrations with rat olfactory bulb extracts (Fig. 2). A maximal primary response occurred 45 days after the first injection and after the booster injection, the anamnestic response was very rapid with a peak in antibody activity within 2 weeks. The antiserum produced 100 μg immunoprecipitate/ml serum at the peak of the primary response and 500 μg/ml at the peak of the secondary response. The antiserum collected for further

TABLE 1

CROSS-REACTIVITY OF OLFACTORY TISSUE EXTRACTS TESTED BY IMMUNODIFFUSION

Species	Olfactory Tissue	Goat Antiserum	Rabbit Antiserum
Rat	Epithelium or bulbs	+	+
Mouse	Epithelium or bulbs	+	+
Hamster	Epithelium or bulbs	+	+
Guinea pig	Epithelium or bulbs	±	±
Pig	Epithelium or bulbs	±	±
Rabbit	Bulbs	±	±
Sheep	Bulbs	±	±
Beef	Bulbs	±	±
Dog	Bulbs	+	+
Goat	Bulbs	-	-
Human	Bulbs	-	-
Chicken	Bulbs	-	-
Frog	Bulbs	-	-
Garfish	Nerve	-	-

The antisera were obtained against the pure olfactory marker protein isolated from the rat olfactory bulbs, as described in Methods. For certain species a specific strong immunoprecipitin line developed within 24 hours (+), for others only a weak, although specific, immunoprecipitin line was observed which took several days to appear (±). For a final group of species no immunoprecipitin line was ever seen (-).

Figure 1. Immunodiffusion analysis of extracts from brain tissue of several species. The specific rabbit antiserum was placed in the central row of wells. The top and bottom rows of wells contain 20 μl of 10 percent extracts from the following tissues: (1) rat epithelium; (2) rat bulbs; (3) mouse epithelium; (4) mouse bulbs; (5) pig epithelium; (6) pig bulbs; (7) sheep bulbs; (8) goat bulbs; (9) guinea pig bulbs; (10) mouse cerebellum. This picture was taken after 5 days at room temperature in a humid chamber. For certain species strong immunoprecipitin lines appeared within 24 hours (——) for other species a weak immunoprecipitin band appeared only after several days (----). In certain cases no precipitate was ever observed.

Figure 2. Formation of specific goat serum antibody following immunization with the pure rat olfactory marker protein. The antibody formation was followed by measuring the amount of immunoprecipitate formed upon addition of rat olfactory bulb extract to the antiserum. The amount of protein precipitated from 1 ml of goat antiserum is plotted against the time after first injecting 500 μg of the pure rat olfactory marker protein (day 0). The arrow indicates the day of the booster injection of 400 μg of pure rat olfactory marker protein. Protein content was determined by the method of Lowry et al (53).

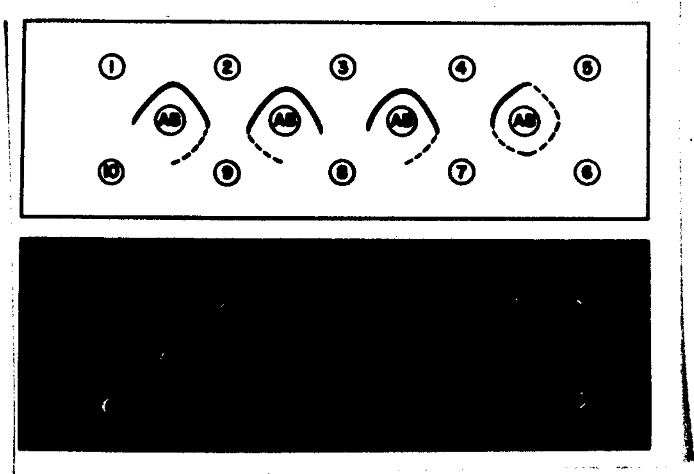


Figure 1

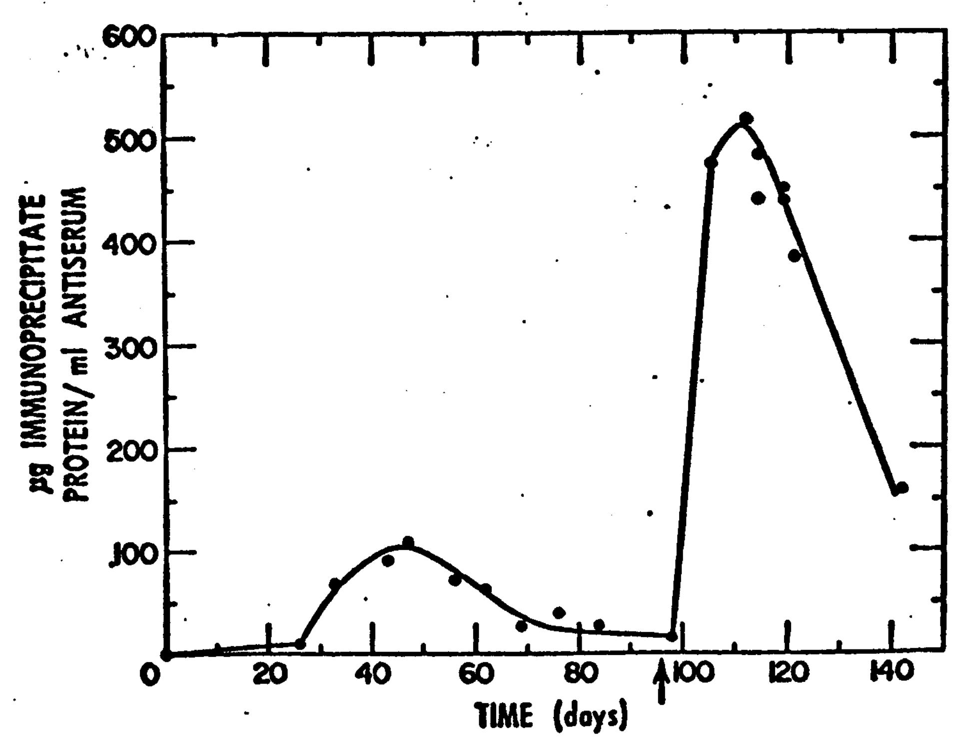


Figure 2

immunoprecipitin tests was drawn 3 weeks after the booster injection, at which time the antiserum produced 450 μg immunoprecipitate/ml. For each immunoprecipitin test, enough tissue extract was used to reach the equivalence point and also to attain antigen excess (Fig. 3, 4).

In agreement with the original report of Margolis (30), a cross-reaction was observed only with the extracts of olfactory tissues. At the equivalence point, about 70 μg of protein was precipitated by 100 μl of antiserum in all the rodent olfactory tissue extracts tested (rat, mouse, hamster, guinea pig). More phylogenetically distant species generally gave smaller protein precipitates at equivalence: 50 μg with the rabbit, 30 μg with the sheep and the cow. Although the dog olfactory bulb extracts did not cross-react with the antibody to the mouse protein (30), in these immunoprecipitin tests they did cross-react and gave 65 μg protein immunoprecipitate at equivalence. The maximal amount of protein precipitated with 100 μl of antiserum was the same for olfactory epithelium and olfactory bulbs of the same species, indicating a probable identity of the protein in both locations.

The cross-reacting protein appears to be present in variable concentrations in olfactory bulbs of different species (Fig. 3, 4). Thus, for the mouse, 140 μg of extract protein were required to reach the equivalence point whereas 200 μg to 500 μg of rat extract protein, 550 μg of guinea pig extract protein and 300-700 μg of hamster extract protein were required. When testing the dog extracts, the equivalence zone was observed to be very broad and to occur between 500 μg and 1100 μg of extract protein (Fig. 4). In the case of the cow, it was possible to dissect the olfactory bulbs into a grey cortical fraction and a white

Figure 3. Immunoprecipitin titration of extracts of olfactory bulbs from several species and rat cerebellum. The amount of protein precipitated from 100 μl of goat antiserum is plotted against the amount of total protein present in the tested extracts. (●) rat, (o) mouse, (▲) hamster, (Δ) guinea pig, (◻) sheep. The rat cerebellum immunoprecipitin curve (■) is typical of tissue extracts from non-olfactory areas of the brain. Protein content was determined by the fluorescamine method. Each immunoprecipitation was performed in duplicate. Mean values are plotted at each point, replicates always agreed, within 15 percent. Each curve is obtained from one experiment and is representative of results obtained with the analysed species. In the case of the hamster (▲) and the sheep (◻) only one experiment was performed.

Figure 4. Immunoprecipitin titration of olfactory bulb extracts from various species and rat cerebellum. Details are as in Figure 3. (●) dog, (o) rabbit (▲ --- Δ) cow; (▲ — ▲) cortical fraction of the cow olfactory bulbs, (◻) rat cerebellum.

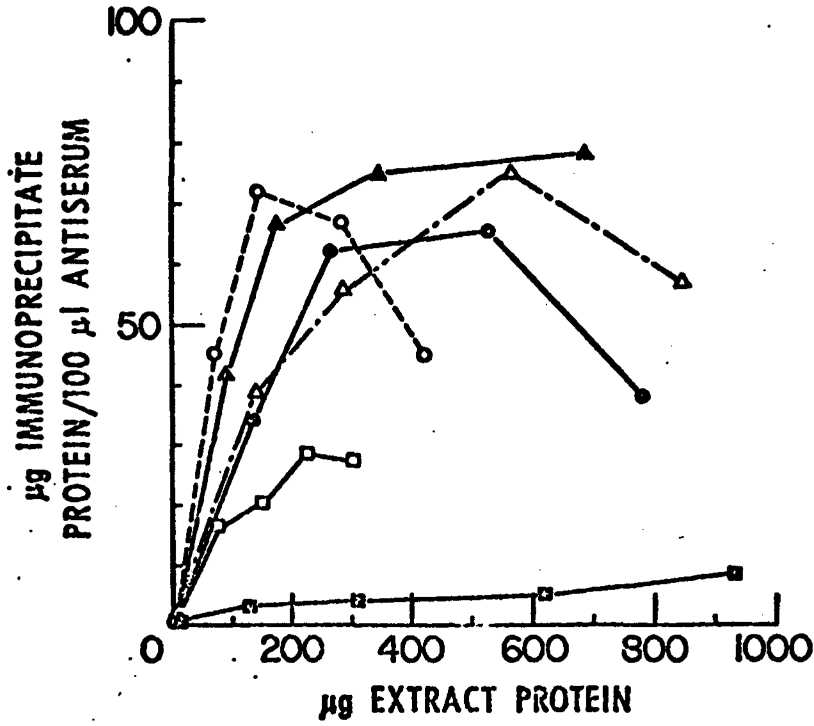


Figure 3

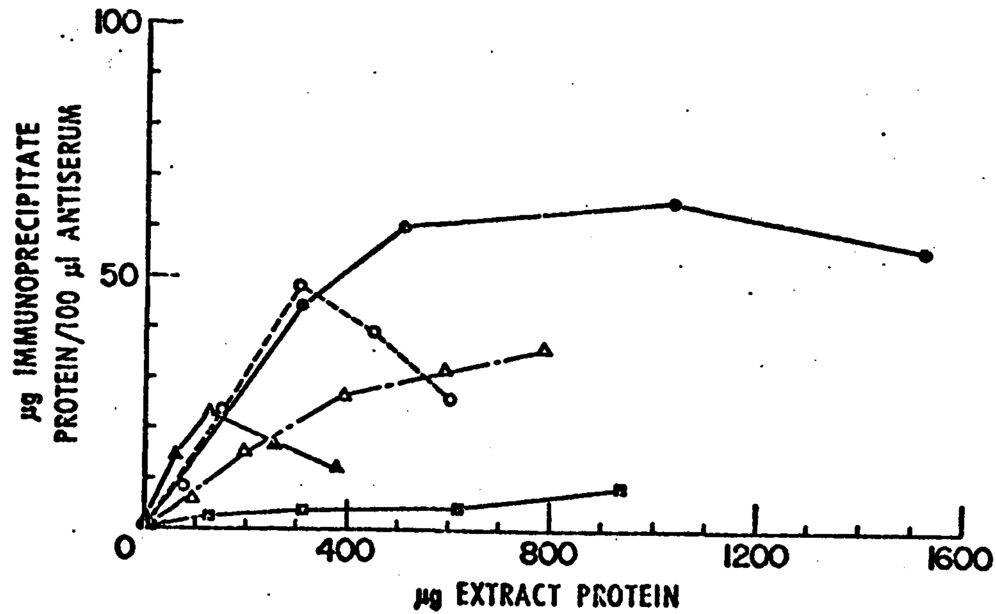


Figure 4

subcortical fraction. The olfactory protein was present primarily in extracts from the cortical portion as shown by the immunoprecipitin test. In these immunoprecipitin titrations, more than 800 μg of extract protein was needed to reach the equivalence zone of the curve when whole cow olfactory bulbs were used but only 120 μg of extract protein when the cortical region of the bulb was assayed (Fig. 4). Extracts of the subcortical fraction were indistinguishable from non olfactory tissue extracts such as cow cerebellum or rat cerebellum. The quantity of immunoprecipitated protein observed at equivalence from the cortical region of cow bulb is two thirds of that observed with extracts of whole bulb. The meaning is unclear.

Human olfactory bulb extracts, assayed by the immunoprecipitin reaction, showed no precipitates which were significantly different from those obtained with extracts of non olfactory areas.

The sensitivity of the immunodiffusion test on agarose plates was comparable to that of the immunoprecipitin titration curve method. Therefore, to test the olfactory tissue extracts of those species which did not cross-react with the goat antiserum by these two methods, we used the much more sensitive radioimmunoassay which could detect as little as 2 ng of the purified rat protein (Fig. 5). As an indication of the reproducibility of the method, titration curves for the pure olfactory protein and for rat olfactory bulb extracts are plotted from 2 different experiments (Fig. 5). The standard curve was linear between 5 and 100 ng of the pure protein. The assay was, therefore, used in that range. The inhibition curve obtained with varying amounts of soluble rat olfactory bulb extract was parallel to the standard curve obtained

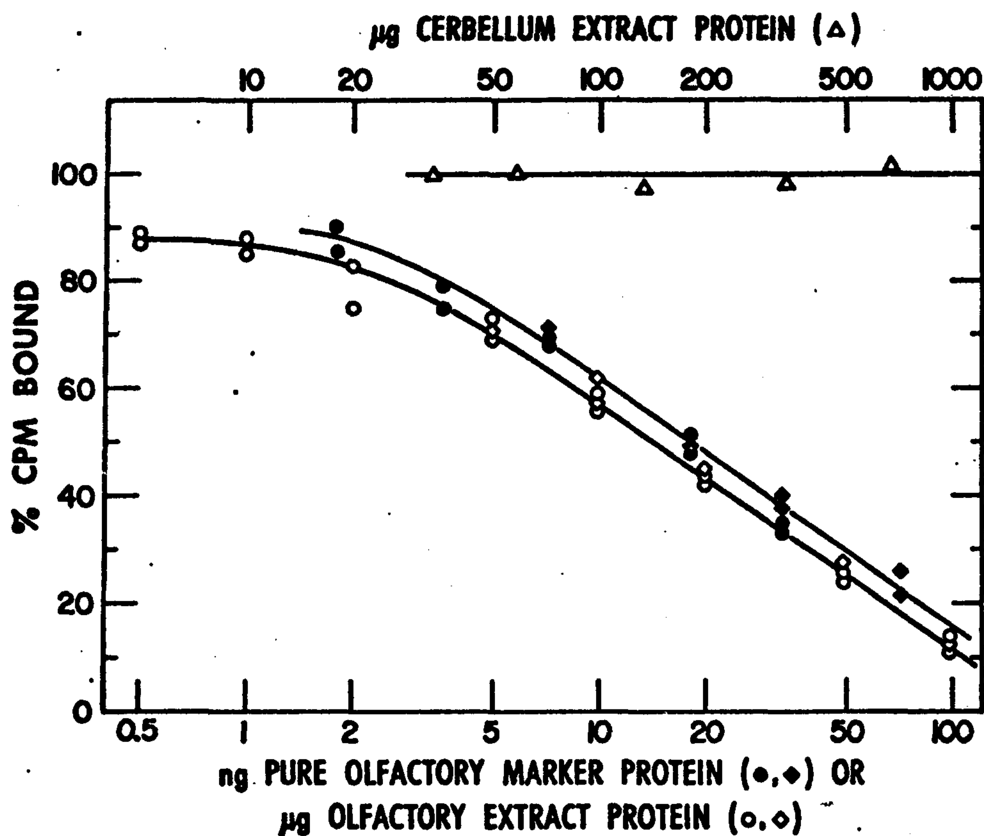


Figure 5. Radioimmunoassay for the rat olfactory marker protein in rat tissue extracts. The percent of counts bound to the antibody coated beads, on the ordinate, is plotted against the ng of pure olfactory marker protein or μg of tissue extract protein on the abscissa. The amount of ^{3}H-labeled tracer bound in the absence of any added protein was taken as 100% (•,♦) rat olfactory bulb extract; (Δ) rat cerebellum extracts; (o,◇) pure rat olfactory bulb marker protein. Data from two experiments have been plotted together to demonstrate the reproducibility of the method. Individual data points are plotted.

using the purified rat olfactory protein and showed that in 5 μg of extract protein, there is about 3.5 ng of the olfactory marker protein. Addition of 50 ng of pure protein to varying amounts of extract protein gave the binding inhibition expected by strict additivity (Table 2). No significant modification in the standard curve was observed when the radioimmunoassay was performed in presence of the other olfactory marker, carnosine (1mM final concentration). The radioimmunoassay was routinely performed using extracts of olfactory tissues from different species. As expected, extracts from species which had previously been shown to cross-react with the goat antibody to the pure rat olfactory marker protein competed with the labeled rat olfactory protein for binding. More interestingly, extracts of olfactory tissues of the additional species, man and garfish, inhibited homogenous rat olfactory protein binding (Table 3). When sufficient frog olfactory bulb extract was used (4700 μg), a lesser but reproducible inhibition of labeled rat olfactory protein binding was observed. No inhibition was ever observed for the chicken olfactory bulb extracts nor for the extracts of rat cerebellum, lung, muscle, and liver even when more than 2000 μg of extract protein was tested.

The binding inhibition curve obtained with varying amounts of garfish olfactory nerve extract (Fig. 6), is parallel to that obtained with the pure rat olfactory protein.

TABLE 2

ADDITIVITY PROPERTIES OF THE RADIOIMMUNOASSAY

FOR THE RAT OLFACTORY MARKER PROTEIN

μg Olfactory Bulb Extract Protein	% Binding Inhibition		
	Alone	In the Presence of 50 ng Additional Marker Protein	
		Observed	Expected
7.3	30	50	51
18.2	45.5	60.5	58
36.5	62.5	69.5	68
73	76.5	76.5	80

To varying amounts of olfactory bulb high speed supernatant extract protein were added 50 ng of pure marker protein. Radioimmunoassays were then performed on these samples and the observed results compared with the expected results calculated from titration curves of extract and purified protein run simultaneously. The results were in agreement with those calculated for strict additivity (% Binding inhibition = 100- % cpm bound).

TABLE 3

SPECIES AND TISSUE DISTRIBUTION OF THE OLFACTORY MARKER PROTEIN

Species	Tissue	μg Extract Protein	% Binding Inhibition
Rat	Olfactory Bulbs	18	40
Hamster	Olfactory Epithelium	10	41
Rabbit	Olfactory Bulbs	54	40
Human	Olfactory Bulbs	750	41
Garfish	Olfactory Nerve	725	40
Frog	Olfactory Bulbs	4700	16
Rat	Cerebellum	3425	0
Rat	Liver	2535	0
Rat	Lung	3025	0
Rat	Muscle	2929	0

High speed supernatant extracts were assayed in duplicate by the competitive binding radioimmunoassay using antibody-coated polystyrene latex beads and ^{3}H-labeled rat olfactory marker protein. The protein contents of the extracts were assayed with fluorescamine (% Binding inhibition = 100- % cpm bound). The % binding inhibition presented is the mean value obtained by interpolation from radioimmunoassay titration curves. The % binding inhibition obtained at the indicated protein concentrations showed a maximum range of 10%, except for the frog extracts for which the range was 15% of the reported value.

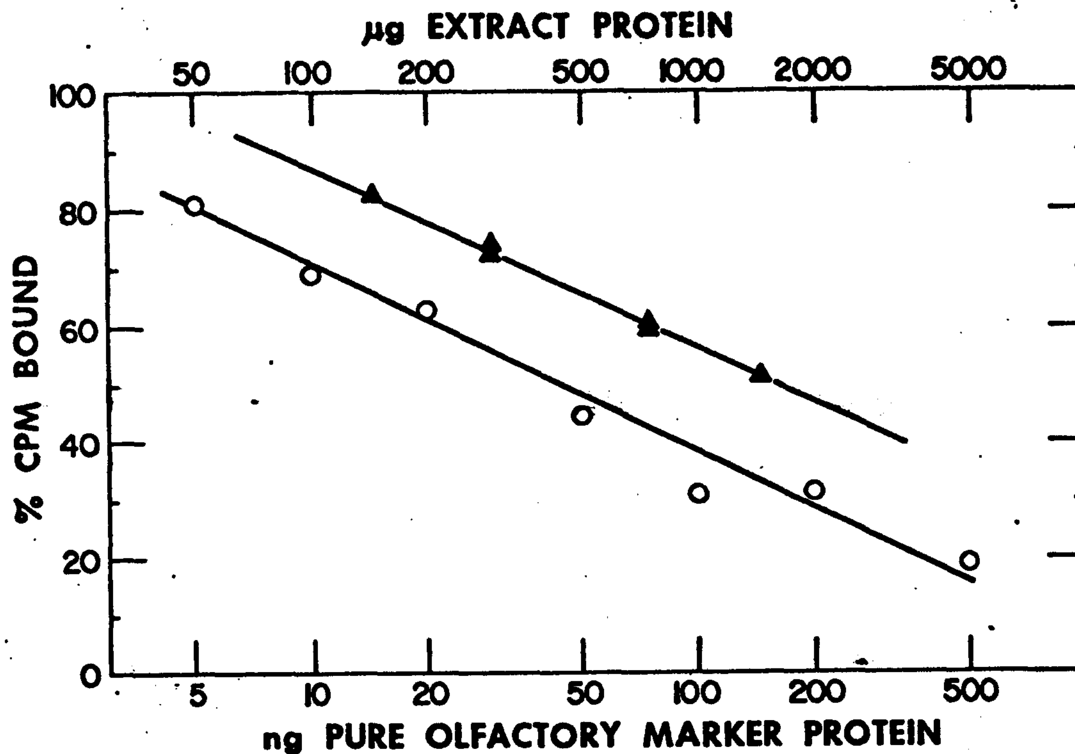


Figure 6. Radioimmunoassay for cross-reactivity of garfish olfactory nerve extracts with antibody directed against the rat olfactory marker protein: Details are as in Figure 5 and present the data from one experiment. (o) pure rat olfactory marker protein; (▲) garfish olfactory nerve extract.

DISCUSSION

The antisera produced by the goat and the rabbit against the purified rat olfactory marker protein demonstrated a very broad species cross-reactivity. On Ouchterlony immunodiffusion plates, a positive specific reaction was observed with extracts of olfactory tissue from representative rodentia, cavidae, lagomorpha, artiodactyla, and carnivora. By quantitative immunoprecipitin tests of these olfactory tissue extracts, it was generally observed that as the taxonomic distance from the rat increases, the protein content in the immunoprecipitate at equivalence is decreased. As the antiserum contains a mixture of antibody molecules with different antigenic specificities, it is likely that only some of these antibody molecules will react with the antigenic determinants of the non homologous antigen. The differences among immunoprecipitin curves of different species presumably indicate structural variations between their respective olfactory marker proteins.

The olfactory marker protein in the extracts of human olfactory bulbs and of garfish olfactory nerves, although unable to provoke a specific immunoprecipitate, are able to bind to the specific antibody and, therefore, to compete with the labeled rat olfactory protein during the radioimmunoassay. The human olfactory bulbs were obtained from autopsy material at least 12-24 hours after death. The poor cross-reactivity of the human olfactory marker protein may well be caused by a degradation of this protein during that time interval and not necessarily by a major structural variation of the native human olfactory marker protein. Carnosine (1 mM) does not interfere with the radioimmunoassay, and, therefore, does not modify the structure of the antigenic

site of the rat olfactory marker protein.

For the cow, about 7 times more of the extract of the whole olfactory bulb than of the cortical fraction of the bulb was needed to reach equivalence. No cross-reaction was observed with the subcortical fraction of the olfactory bulbs. This indicates that the marker protein is concentrated in the cortical area which contains the nerve endings of the olfactory chemoreceptor neurons. The amount of immunoprecipitate obtained with whole olfactory bulbs extract is somewhat more than that obtained with the cortical fraction. The cause of this discrepancy is unclear. Thus, the apparent very low cross-reactivity of the frog extracts, or non cross-reactivity of the chicken extracts may only be a problem of concentration, since we experienced difficulty dissecting the very small olfactory bulbs of these species. By contrast, the olfactory nerve is a rich source of olfactory protein since it consists mainly of the axons from the very cells that synthesize and contain the olfactory protein. This could explain why much less garfish olfactory nerve extract is needed to see a more extensive binding inhibition than from the frog olfactory bulb extracts. Thus, the list of the species possessing the olfactory marker protein has been extended to include primates, amphibians, and teleosts. The sensitive radioimmunoassay demonstrated not only the very broad species distribution of the olfactory marker protein, but also the restricted specificity of this marker for the primary olfactory pathway. No olfactory marker protein could be detected in the rat cerebellum (as an example of other brain region), lung (as an example of other respiratory epithelium), muscle (as an example of other excitable tissue) or liver.

The data presented in this chapter demonstrates that this protein is characteristic of olfactory function and is present in all vertebrate animals. One would, therefore, expect to find this protein or an analogous protein in all such animal groups. However, its absence in non-vertebrate species cannot be ruled out by these experiments. The protein is an excellent biochemical marker of the olfactory chemosensory neuron. It should facilitate the development of biochemical investigations of this sensory system, about which very little is known.

The antibody itself could also be used for immunohistofluorescence studies in order to localize more precisely the protein in situ. It is possible that irrigation of the epithelium or the olfactory bulb with antibody would provoke an antigen antibody reaction resulting in damage to the specific cell. If all the chemosensory neurons are thus killed, one would have performed a specific neuronal destruction analogous to the use of antibody to NGF to produce an immunosympathectomy (56). This would be very useful, since it would obviate surgical side effects. Since the rabbit produces antibodies to the rat protein which cross-react against its own olfactory marker protein, and γ-globulin can cross the placenta (57), it would be theoretically possible to obtain an immunized pregnant rabbit producing antibodies against the olfactory receptor cells of its own offspring which would then be congenitally anosmic and serve as a model for behavioral and biochemical studies.

ACKNOWLEDGMENTS

I thank Drs. R. A. Rush, S. H. Kindler, and S. Udenfriend for kindly permitting me to read their manuscript prior to publication.

(II) ISOLATION AND CHARACTERIZATION OF THE OLFACTORY MARKER PROTEIN FROM RAT OLFACTORY BULBS

Following the fruitful attempts of W. B. Moore to isolate "any protein specific to the nervous system" (27), several proteins specific to the mammalian central nervous system have been discovered in recent years. These proteins of unknown functions represent differential genetic expression of the neurons and glia. In addition to small acidic proteins such as S100 (58) and GFA (60) which are specific to glial cells or such as 14.3.2 (58, 59) which is specific to neurons, glycoproteins have also been isolated. These glycoproteins include GP-350 (61) which is neuron specific, partly soluble, and partly membrane bound, the α_2 glycoprotein (62) which is glial specific,and the sialoglycoprotein 10-B (63) which is restricted to astrocytes. Moreover, nervous system specific cell surface markers (NS1 and NS2) have also been isolated from glioblastoma and shown to be present only in glial cells (64). These proteins are organ specific and are present, although in variable concentrations, in every area of the brain. Therefore, they must be representative of general functions of the neurons or the glia in the same way as the enzymes involved in neurotransmitter metabolism are characteristic of the neurons which utilize those transmitters. Nervous system proteins of more restricted location and, therefore, representative of more specific functions, have been detected in invertebrates and in vertebrates. In invertebrates, specific proteins, glycoproteins, or polypeptides are synthesized by identified neurons (65-68). In higher vertebrates, the neurosecretory cells of the hypothalamus and of the pituitary synthesize

polypeptide hormones (69). Hydroxyindole-O-methyl transferase is a marker for the melatonin containing cells of the pineal (70). Few other brain regions or pathways have been analyzed for specific proteins. There is evidence for a specific protein to the cell membrane of the optic tectum which disappears upon optic nerve section (71). In the central areas of the auditory pathway, gel electrophoretic analysis indicates that there are specific proteins to certain cerebral regions associated with the processing of auditory information (72). However, these proteins have not been isolated and characterized.

Immunological studies with antisera against the rat olfactory marker protein showed that the olfactory marker protein, specific to the primary olfactory pathway (45, 46, 73),has indeed a very wide species distribution and that it is probably present in all vertebrates (Chapter I). Therefore, this protein very probably has an important role related to the function of the olfactory receptor cells. This chapter will describe the isolation and characterization of the rat olfactory marker protein and compare its physicochemical properties to those of the mouse protein.

EXPERIMENTAL

Materials: Male Sprague-Dawley derived rats (5-6 weeks old) were killed by exsanguination after nembutal injection. The olfactory bulbs were then removed, used fresh, or stored at -80°C. Frozen olfactory bulbs from rats of mixed ages and sexes were also purchased from Dr. A. F. Parlow, Harbor General Hospital, Torrance, California and stored at -80°C. Frozen garfish bills were purchased from Gulf Specimen Company, Inc., Panacea, Florida. Ampholines pH range 3-10 and 4-6 were obtained from LKB Produkter AB Sweden (40% solution). Dimethylsuberimidate dihydrochloride was purchased from Eastman Kodak Company, Rochester, New York. Fluorescamine was obtained from Roche Diagnostics, Nutley, New Jersey. All other materials were of analytical reagent grade.

Purification of the olfactory marker protein: The rat olfactory marker protein was first isolated by a procedure similar to that already described for the mouse protein (30). Recently, a modified procedure has been utilized. Both of these procedures will be described. Initially, the radioimmunoassay described by Margolis (49) for the mouse protein was used to monitor the isolation of the rat olfactory marker protein. This was possible because the goat antibody to the mouse olfactory marker protein cross-reacts with the extracts of rat olfactory tissues (30). Purified mouse olfactory protein was used as a standard. In subsequent studies, this heterologous radioimmunoassay was replaced with a more sensitive homologous radioimmunoassay. This modified radioimmunoassay utilizes the goat antiserum directed against the rat olfactory marker protein. Purified rat olfactory protein is used as the standard. This

assay, which has a very high specificity and broad species cross-reactivity, has been described in detail in the previous chapter. Total protein was initially measured by the method of Lowry et al. (53) and in later experiments by the more sensitive method using Fluorescamine (52).

A. Initial preparation. All steps were carried out at 0-4°C in 0.01 M sodium phosphate buffer pH 7.7. Aliquots were kept for analysis at each step of the purification. Frozen bulbs from 330 rats were pooled (20 g) and homogenized with 100 ml of the cold buffer. The homogenate was centrifuged for 1 hour at 100,000 g and the supernatant recovered. Stepwise, ammonium sulfate precipitation was performed as previously described (30). The protein fraction which precipitated between 63% and 95% saturated ammonium sulfate was dialyzed overnight against a large volume of distilled water. The dialyzed protein fraction was adjusted to pH 7.7 with sodium phosphate buffer, 0.01 M final concentration (100 ml) and was chromatographed on a DEAE-cellulose column (2 x 25 cm) equilibrated with the same buffer. The flow rate was 30 ml per hour and 5 ml fractions were collected. Elution was first carried out with the phosphate buffer (130 ml) until A_{280} of the eluate fell below 0.100. Elution was then continued with a linear salt gradient formed from 200 ml of buffer in one chamber and 200 ml of buffer containing 0.08 M NaCl in the second chamber. The salt gradient was monitored by measuring the conductivity of each fraction. Radioimmunoassay was performed on 100 μl aliquots to locate the olfactory marker protein. Fractions containing the rat olfactory marker protein were combined (65 ml). The solution of purified rat olfactory marker protein thus obtained was lyophilized, redissolved in a small

volume of distilled water, submitted to dialysis against two 3 liter changes of distilled water, filtered through a Millipore filter (0.22 μ), and subsequently stored at -80°C.

B. Modified preparation. Two main modifications were made in the initial preparation. One derived from the observation that zinc sulfate precipitated many proteins of the extract, while leaving the olfactory marker protein in solution. The other modification was the use of preparative isoelectrofocusing before DEAE cellulose chromatography. All steps were carried out at 0-4°C. Rat olfactory bulbs (40 g) were homogenized with 9 vol of 5×10^{-4} M EDTA in 5×10^{-3} M Tris HCl buffer at pH 7.4. The crude homogenate was centrifuged for 90 min at 60,000 g with a 40.2 Spinco rotor. Zinc sulfate was added to the supernatant (335 ml) to a final concentration of 2 mM. After stirring for 30 min, the resulting suspension was centrifuged again for 90 min at 60,000 g, and the supernatant (320 ml) was lyophilized, resuspended in distilled water to a final volume of 32 ml and dialyzed against 4 liters of H_2O. The conductivity in the external chamber was monitored every 15 minutes and reached a plateau in one hour at which time the dialysis bath was changed. After 4 such dialysis bath changes, the turbid solution was centrifuged for 10 min at 27,000 g. The final supernatant (35 ml) was kept frozen overnight and submitted to preparative isoelectrofocusing the following morning. A modification of the Valmet Method (74) developed by Dr. W.D. Denckla (manuscript in preparation US Patent No. 3, 901, 780) was used. In this method, the proteins do not migrate vertically in one column but horizontally between 10 chambers in series. The anode and cathode compartments form 2 additional chambers at the extremities of this machine. Ampholines (pH

4-6) were prerun in order to prepare ampholines of a narrower pH range (4.6-5.3). The prerun ampholines were added to the olfactory marker protein preparation to achieve an ampholine concentration of 0.25% in a final volume of 230 ml. The precipitate formed on the addition of the ampholines was removed by centrifugation for 10 min at 27,000 g. The supernatant was submitted to the preparative isoelectrofocusing procedure. The voltage was 10 KV with a current of 15 ma at the beginning of the run, and the voltage was increased slowly to 25 KV at the end of the run. After 3 hrs, the current remained constant at 7 ma. After 2 more hours, the isoelectrofocusing run was stopped, and the fractions were collected from each of the chambers (20 ml per chamber). The temperature was maintained between 5-10°C throughout the run. The olfactory marker protein was localized by the radioimmunoassay, and the pH of each fraction was determined. Among the advantages of this preparative isoelectrofocusing method are a higher capacity, a shorter separation time, and less diffusion than with the usual column isoelectrofocusing procedure.

The pH gradient obtained through the 10 chambers ranged from pH 11.7 to pH 2.5. The olfactory marker protein was distributed among 4 chambers, from pH 4.9 to 5.35. They were pooled, and the pH was adjusted to neutrality by addition of 0.1 M sodium phosphate buffer at pH 7.7 to 0.01 M final concentration. This fraction (85 ml) was then chromatographed on a DEAE-cellulose column under conditions similar to those described for the initial preparation. However, the eluting gradient was formed from 300 ml of buffer in one chamber and 300 ml of buffer containing 0.1 M NaCl in the second chamber. Elution was further continued with 100 ml of buffer containing 1 M NaCl in order to remove any olfactory marker protein which

could have been retained on the column. Radioimmunoassays and total protein determinations were as described above on aliquots of the 5 ml fractions.

Polyacrylamide disc gel electrophoresis: Polyacrylamide disc gel electrophoresis was performed following the method of Davis (75) as previously described (30). We used 10 cm long running gels consisting of 14% acrylamide and 0.37% N,N'-methylene bis acrylamide, polymerized with 0.35% ammonium persulfate. The 1.5 cm long stacking gels were photopolymerised with riboflavin. The samples, 0.1 ml - 0.2 ml in 20% sucrose, were loaded by buffer displacement and electrophoresis was run for 3 hours at 3 mA per tube. Gels were fixed and stained overnight with 0.05% Coomassie blue in methanol-glacial acetic acid-water (20:7:13) and excess stain was removed by extensive washing with the same solvent.

The isoelectric point of the protein was analyzed by the technique of isoelectrofocusing on polyacrylamide gels, as described by Wrigley (76). The gel mixture contained 1% carrier ampholines, pH range 3-10. Gels (5 mm x 6 cm) were loaded with about 20 μg of the protein to be analyzed and stained with Coomassie blue after removing the ampholines by soaking in 5% Trichloroacetic acid. The pH gradient was determined as follows: 3 gels without protein and run simultaneously were sliced into 1 mm pieces. Fractions consisting of 2 slices each were incubated overnight in 1 ml of distilled water. Their pH was then measured, and the pH gradient along the gel plotted and compared to the normalized mobility of the protein.

The molecular weight of the rat olfactory marker protein was estimated by the technique of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) as described by Weber and Osborne

(77). The samples (5-20 μg protein) were dissolved in 180 μl of 0.01 M sodium phosphate buffer pH 7.0, containing 1% SDS and 1% β-mercaptoethanol and incubated for 20 minutes at 85°C. After adding sucrose (10% final concentration) to the samples, they were loaded on the gels (5 mm x 6 cm) by buffer displacement and electrophoresed for 3-4 hours. Some protein samples were pretreated with a cross-linking reagent, following the method described by Davies and Stark (78). These protein samples were incubated for 3 hours at room temperature with dimethylsuberimidate dihydrochloride, 1 mg/ml in 0.2 M triethanolamine hydrochloride, pH 8.5, and subsequently submitted to the usual procedure for SDS gel electrophoresis.

Gel filtration on Biogel P-60: A column (2 x 36 cm) of Biogel P-60 (Biorad) was equilibrated with 0.01 M sodium phosphate buffer pH 7.7 containing 0.15 M NaCl. The flow rate was 20 ml/hr,and 1 ml fractions were collected. The void volume (Vo) was estimated as the elution volume of dextran blue (Pharmacia). The molecular weight of the specific protein was estimated by comparison of the ratio $\frac{Ve}{Vo}$ obtained in this case to the values obtained for standard proteins where V_e represents the elution volume of the various proteins.

Gel filtration on Sephadex G-75: A column (1 x 58 cm) of Sephadex G-75 was equilibrated with 0.01 M sodium phosphate buffer pH 7.7 containing 0.2 M NaCl. The flow rate was 11 ml per hour,and 0.5 ml fractions were collected. The molecular weight was estimated as described for the Biogel P-60 column.

Filtration on concanavalin A sepharose column: To determine whether the olfactory marker protein contained any carbohydrate, a concanavalin A

Sepharose column (1.5 ml bed volume) was equilibrated with 0.1 M sodium phosphate buffer pH 7.0 containing 0.1 M NaCl. The elution of the column was performed with the same buffer solution containing 10% of α-methyl-D-glucose or a α-D-mannoside.

Amino acid analysis: The homogenous protein obtained by the modified procedure was analyzed according to the method of Stein *et al.* (79). The pure protein (2 μg) was hydrolyzed in 6 N HCl for 24 hrs. The hydrolysis and amino acid analysis were performed in duplicate. Tryptophan content was determined by the method of Edelhoch (80), by measuring the absorbance of 280 mμ of 180 μg of pure rat olfactory marker protein dissolved in 1 ml of 6 M guanidine hydrochloride.

Stability of the rat olfactory marker protein:

A. The immunological activity of the rat olfactory marker protein in pure form or in high speed supernatant of the crude extract was tested following dialysis against distilled water, against different buffer solutions, and following storage at 0-4°C or at -20°C for variable durations of time. The pellet obtained during the purification of the olfactory marker protein after precipitation by 95% ammonium sulfate was resuspended (P_{95} fraction) and submitted to these tests.

B. Thermal denaturation: a solution of the pure rat olfactory marker protein in distilled water was used in these studies. It contained 500 ng of the immunologically active protein per 10 μl and as estimated by the homologous radioimmunoassay. To 200 μl of the appropriate solution, 10 μl of the purified rat olfactory marker protein was added. Samples were incubated for variable durations of time in a boiling water bath or in a 37°C water bath. At the end of the treatment, the tubes were placed on ice and

total protein content as well as immunologically active protein were estimated.

RESULTS

Purification of the rat olfactory marker protein. Initial Preparation: The rat olfactory marker protein was purified nearly 100 fold during the initial preparation (Table I) with a recovery of 60% of the immunological activity of the marker protein. The rat olfactory marker protein was eluted from the DEAE-cellulose column at 0.04 M NaCl in a peak well separated from the bulk of the soluble pvoteins (Fig. 1). From this preparation, by radioimmunoassay, we obtained 1174 μg of marker protein for use in further analysis. A homogeneous preparation of the rat olfactory marker protein should have a specific activity of 1000 μg marker protein per mg total protein, by definition. However, the specific activity attained by this preparation was never seen to exceed 300 μg/mg. This may reflect the use of the heterologous mouse radioimmunoassay which gives a relative but not absolute measurement of the quantity of the rat olfactory protein. The protein sample collected from the DEAE cellulose column was lyophilized, resuspended in 1 ml distilled water, and dialyzed against distilled water. During this dialysis, a loss of immunological activity was always observed. Thus, the final recovery of rat olfactory marker protein, as estimated by the radioimmunoassay, was only of 25%.

Polyacrylamide gel disc electrophoresis. The purity of the protein eluted from the DEAE-cellulose column was analyzed by polyacrylamide gel disc electrophoresis. Scanning of the gel at 255 mμ indicated that the protein was 80% pure. A comparison of the patterns obtained with a crude extract and the purified fraction of marker protein is shown in Fig. 2.

TABLE I

	Volume ml	Protein mg	Olfactory Marker Protein µg	Percent Recovery	Specific Activity*	Purification
High speed supernatant	95	565	1937	100	3.4	1
63 percent ammonium sulfate supernatant	245	130	1507	77.8	11.6	3.4
63.95 percent ammonium sulfate precipitate	23.5	136	1600	82.6	11.7	3.4
DEAE cellulose eluate: pooled tubes 32-44	87	3.9	1174	60.6	301	88.5

Purification of rat olfactory marker protein. Initial preparation - The amount of rat olfactory marker protein in the sample was estimated by the heterologous radioimmunoassay. The amount of total protein in the sample was estimated by the method of Lowry.

*Specific activity is calculated as µg marker protein divided by mg of total protein.

Figure 1. Elution profile from DEAE cellulose column.

▲ ▲ ▲ Salt gradient

●-●-● Rat olfactory marker protein estimated by the radioimmunoassay as percent binding inhibition of the mouse protein (31).

o o o Total protein estimated by the method of Lowry.

Figure 2. Polyacrylamide gel patterns.

1. Rat olfactory bulb high-speed supernatant (200 μg total protein).
2. Purified rat olfactory marker protein - initial preparation (30 μg total protein).

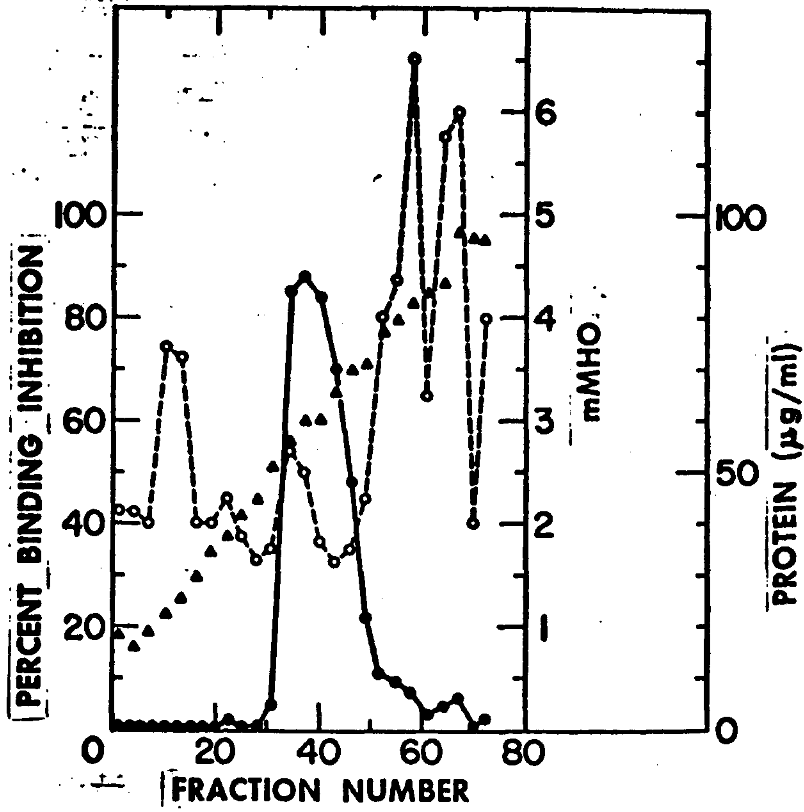


Figure 1

Figure 2

Several faint bands of contaminating protein were visible when 30 μg of purified protein was electrophoresed but a considerable purification of the specific protein has obviously been achieved. Isoelectrofocusing and SDS gel disc electrophoresis (Fig. 3, Fig. 4) of the purified fraction where lower amounts of protein were electrophoresed show only the one band of specific protein.

When the rat and mouse olfactory marker proteins were mixed together before loading on the isoelectrofocusing gel, they were well separated during the run indicating different isoelectric points for these 2 proteins (Fig. 3). Bovine serum albumin exhibited an intermediate migration rate. Analysis of the pH gradient formed in the gel permits the determination of the isoelectric point (pI) of the analysed protein from its relative mobility. The rat olfactory marker protein has a pI of 5.0, in contrast to the mouse protein which manifest a pI of 4.7. In this system, bovine serum albumin has a pI 4.85 - 4.9 in agreement with published values (81).

Analysis of the relative mobility of standard proteins of known molecular weight on SDS gel electrophoresis permitted the estimation of the molecular weight of the rat olfactory marker protein (Fig. 4). We found that the olfactory marker protein migrates as a single band located between Ribonuclease A (13,700) and Chymotrypsinogen (25,000). Estimation of the molecular weight using the semilogarithmic plot of the relative mobility (Rf) against the molecular weight gave a value of 16,500 ± 1500 (mean value from 3 experiments). The mouse olfactory marker protein was indistinguishable from the rat marker protein by this technique. The results were identical when either marker protein was pre-incubated in SDS

Figure 3. Isoelectrofocusing gel electrophoresis. From right to left: 10 μg rat olfactory marker protein, 10 μg mouse olfactory marker protein; 10 μg of rat and mouse marker protein; 10 μg BSA.

Figure 4. Molecular weight estimation by electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate.

1. Ovalbumine (45,000), chymotrypsinogen (25,000), pancreatic trypsine inhibitor (8500).
2. BSA (67,000), chymotrypsinogen, ribonuclease A (13,700).
3. Chymotrypsinogen, rat olfactory marker protein (10 μg).
4. Rat olfactory marker protein (10 μg).

Figure 3

Figure 4

solution with or without β-mercaptoethanol present. Preincubation of the rat olfactory marker protein in the presence of the crosslinking agent dimethylsuberimidate hydrochloride did not give additional bands corresponding to multiple molecular weights. In contrast, hemoglobin treated in the same manner did show new protein bands corresponding to the dimer, trimer, and tetramer (Fig. 5).

Molecular weight determination by gel filtration techniques. The molecular weight of the native marker proteins were estimated by filtration of crude extracts through a column of Biogel P-60 or a column of Sephadex G-75. The same results were obtained with either of these columns. The marker protein was detected in the eluate by radioimmunoassay, except when the protein had been tritium labeled, in which case the radioactivity was counted directly.

The rat olfactory marker protein present in olfactory bulb extracts had an elution volume corresponding to a molecular weight of 30,000 daltons (Fig. 6). Gel filtration of rat olfactory epithelium extracts gave results identical to those obtained with the olfactory bulb extracts. The immunologically cross-reactive protein in mouse olfactory bulb and epithelium extracts also had an identical elution volume. However, pure marker protein isolated from the mouse or rat olfactory bulbs and labeled with tritium (49, Chapter I) could be distinguished from the native olfactory marker protein present in extracts during this filtration. Furthermore, unlabeled pure rat olfactory marker protein had the same elution volume as the labeled rat and mouse pure proteins, corresponding to a molecular weight of 35,000 daltons. Garfish olfactory nerve extract prepared in the same manner as the rat extract was analyzed on

Figure 5. Molecular weight estimation by electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate. The standard proteins are as in Figure 4 and represented by a full circle. The arrows indicate the relative mobility of the bands obtained with pure olfactory marker protein, ROMP (10 μg) and hemoglobin Hb (8 μg) after dimethylsuberimidate treatment.

Figure 6. Elution pattern from the native and purified olfactory marker protein from Biogep P-60. o —— o purified ^{3}H-labeled marker protein. ● —— ● olfactory bulb extract. The olfactory marker protein was evaluated by radioimmunoassay.

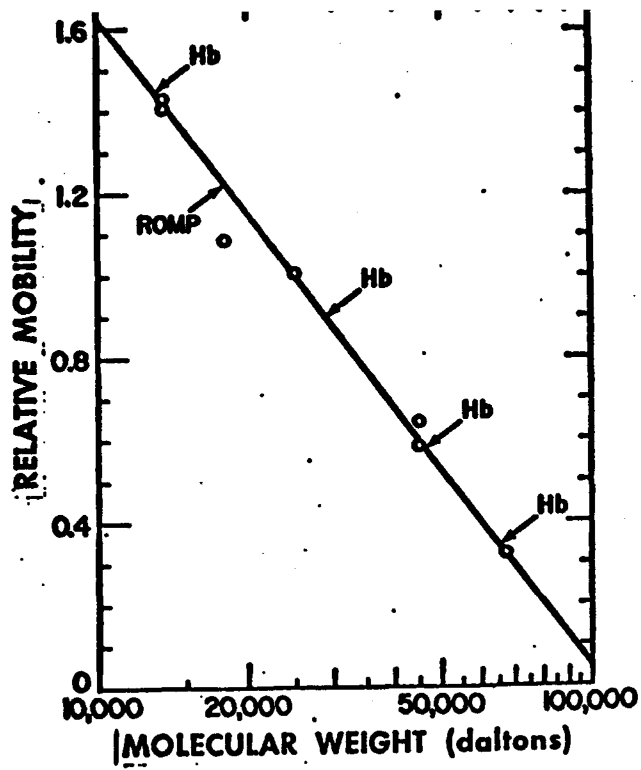


Figure 5

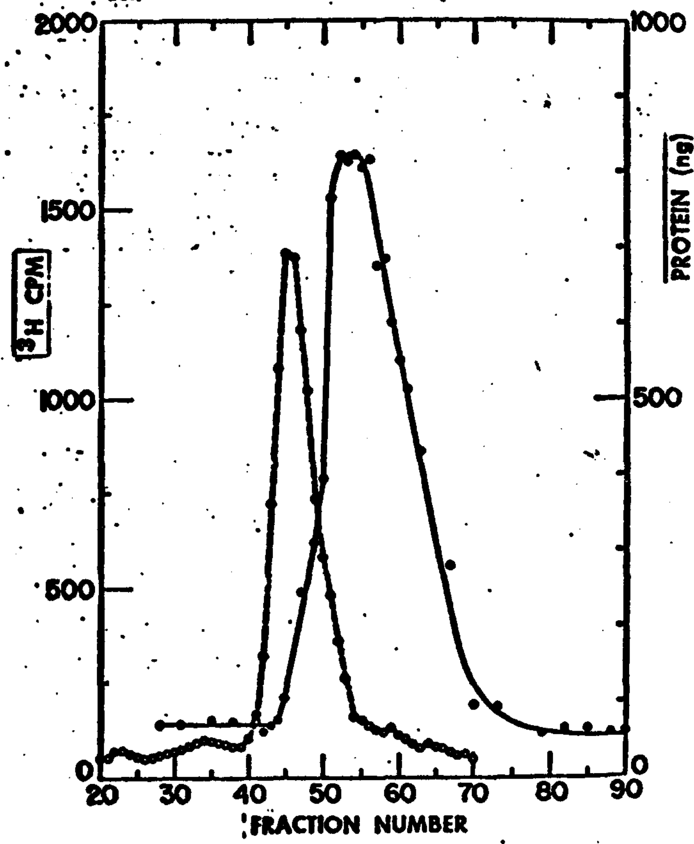


Figure 6

the Sephadex G-75 column. The molecular weight of the garfish olfactory marker protein as determined with the radioimmunoassay was estimated to be 30,000 daltons (Fig. 7). Two other elution peaks were collected when pure, labeled, or unlabeled rat olfactory marker protein was chromatographed. A small peak of immunologically active protein was eluted in the void volume (Fig. 8). A large peak of non immunologically active material was eluted after the main peak of pure rat olfactory marker protein. It was visible by monitoring the radioactivity in the eluate, after chromatography of tritiated pure protein or by monitoring fluorescamine reactive compounds after chromatography of unlabeled pure protein. This peak eluted as a low molecular weight species. Results obtained with protein samples from initial and modified preparations were identical.

Concanavalin A sepharose column. After loading the column with 0.5 ml of 10% rat olfactory bulb extract and rinsing with the phosphate-buffered saline, 120% of the marker protein immunological reactivity was recovered. Upon further elution of the column with 10% α-methyl-D-glucose or α-D-mannoside, no marker protein was eluted. The same procedure was performed on other tissue extracts and dopamine-β-hydroxylase and acetylcholinesterase were shown to be retained by the column and to be eluted by the α-methyl-D-glucoside or α-D-mannoside.

Purification of the rat olfactory marker protein.Modified preparation. To obtain more highly purified protein for amino-acid analysis and additional physical chemical studies, a modified preparation was developed using a preparative isoelectrofocusing step. The nearly homogenous protein (Fig. 9) was obtained in 30% yield (Table 2). A small loss of immu-

Figure 7. Elution pattern of the olfactory marker protein from Sephadex G-75. Elution volumes were calculated relative to Dextran Blue and for standard proteins are represented by the full circles. The standards used and their molecular weights are egg-white trypsin inhibitor (6500); pancreatic trypsin inhibitor (8500); cytochromic (12,300); ribonuclease (13,700); chymotrypsinogen (25,700); ovalbumin (45,000); bovine serum albumin (67,000). Extract of garfish olfactory nerve at 1; extract of rat olfactory bulb and olfactory epithelium at 2; [^{3}H]-labeled purified mouse and rat olfactory proteins at 3; purified rat olfactory protein before and after ^{3}H-labeling at 4.

Figure 8. Elution pattern of the tritiated olfactory marker protein from Sephadex G-75. Three peaks are visible corresponding to the void volume (fraction 13-14), to the immunologically active protein (fraction 21-22) and to non immunologically active material (fraction 34-38).

Figure 9. Polyacrylamide gel pattern from various steps of the modified purification. (a) From left to right 1, 2, 3, 4 are from fractions collected from the isoelectrofocusing chambers at pH 5.35, 5.25, 5.05, 4.9 respectively. 5 is from the pooled fraction obtained from the isoelectrofocusing step, and 6 from the fraction obtained after DEAE cellulose chromatography. On each gel, 60 μg protein were used except 6 (10 μg). (b) From left to right 10 μg and 50 μg rat olfactory marker protein.

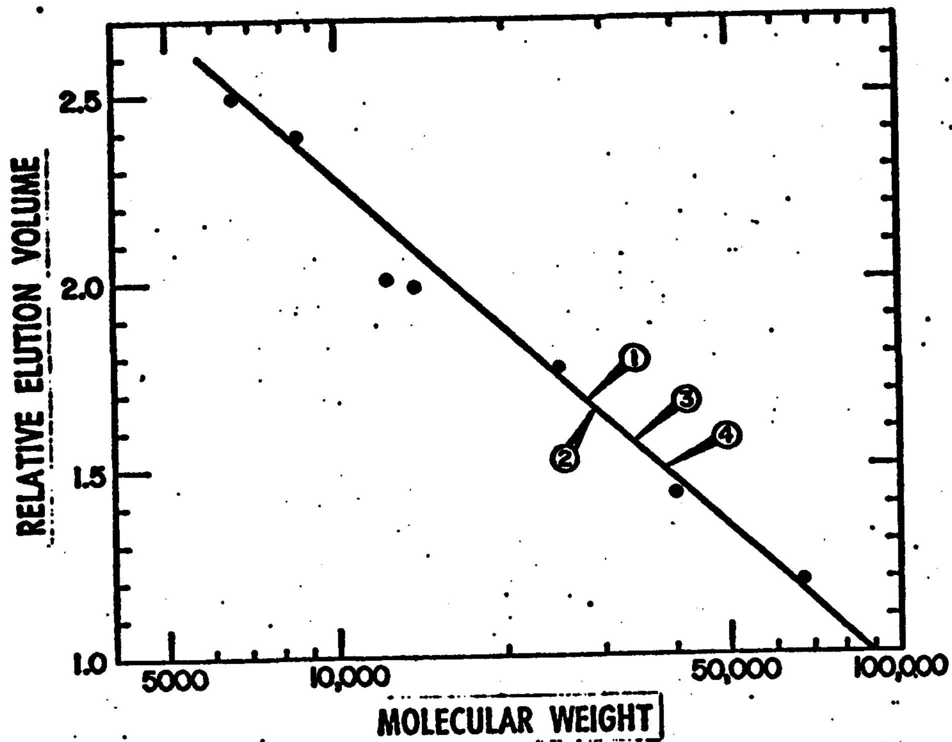


Figure 7

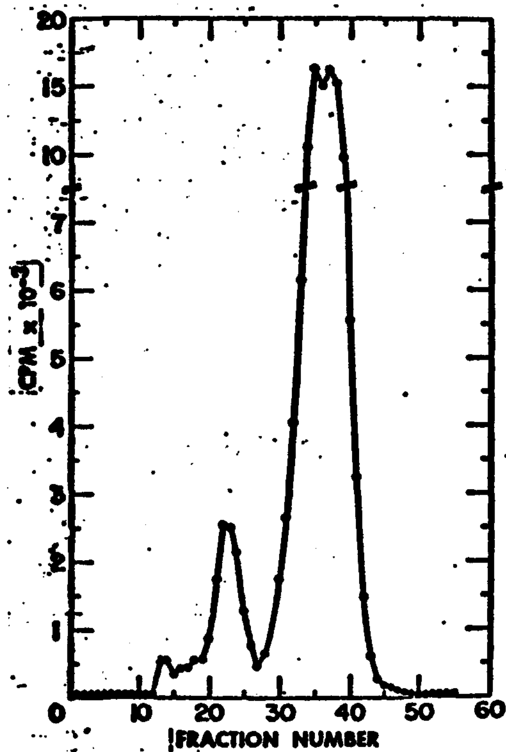
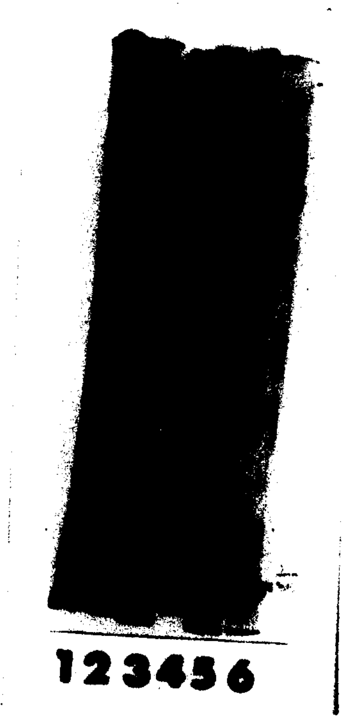


Figure 8



a.

b.

Figure 9

TABLE II

	Volume ml	Protein mg	Olfactory marker protein μg	Percent Recovery	Specific Activity*	Purification
High speed supernatant	330	1515	2891	100	1.9	1
High speed supernatant, following $ZnSO_4$ precipitation and lyophylization	35	278	2016	70	7.3	3.8
Fraction collected after isoelectrofucusing	89	95	1067	37	11.2	5.8
DEAE cellulose eluate pool III	212	4.7	940	32.5	200	105

Purification of the rat olfactory marker protein. Modified preparation - The amount of rat olfactory marker protein in the sample was estimated by the homologous radioimmunoassay. The amount of total protein in the sample was estimated by the Fluorescamine method.

*Specific activity is calculated as μg marker protein divided by mg of total protein.

nological activity occurred after dialysis of this protein, bringing the final recovery to 25%. Only a few faint contaminating bands were visible on Ornstein-Davis polyacrylamide gel electrophoresis, even when 50 μg of the protein was loaded per gel. It is not clear why a protein virtually homogeneous by gel electrophoresis manifests a specific activity of only 200-300 μg/mg rather than the anticipated value of 1000 μg/mg. The main advantage of this preparation was to yield a purer protein for amino acid analysis and other molecular weight analysis. However, modification in the tertiary structure of the marker protein does seem to have occurred since the elution pattern from the DEAE-cellulose column (Fig. 10) now was different than in the original preparation. The marker protein was eluted in 2 peaks. The first, minor peak corresponded to the main peak of the initial preparation, in which 13% of the protein was now eluted by 0.04 M NaCl. Most of the rat olfactory marker protein (80%) was now eluted by 0.08 M NaCl. No more olfactory marker protein could be eluted by 0.1 M NaCl. Although this elution pattern had been modified, the other physicochemical characteristics of this purified protein were identical to those of the rat olfactory marker protein isolated by the initial preparation. The isoelectric point (5.0), the mobility on Ornstein-Davis gel electrophoresis, and the apparent molecular weight on Sephadex G-75 (35,000 before and after tritium labeling) were unchanged. This pure protein was tritiated and used in the homologous radioimmunoassay (Chapter 1). Pure rat olfactory marker protein prepared in the same way was used as a standard in this assay.

Amino acid composition. The amino acid analysis of the rat olfactory marker protein was performed in triplicate with a protein sample obtained

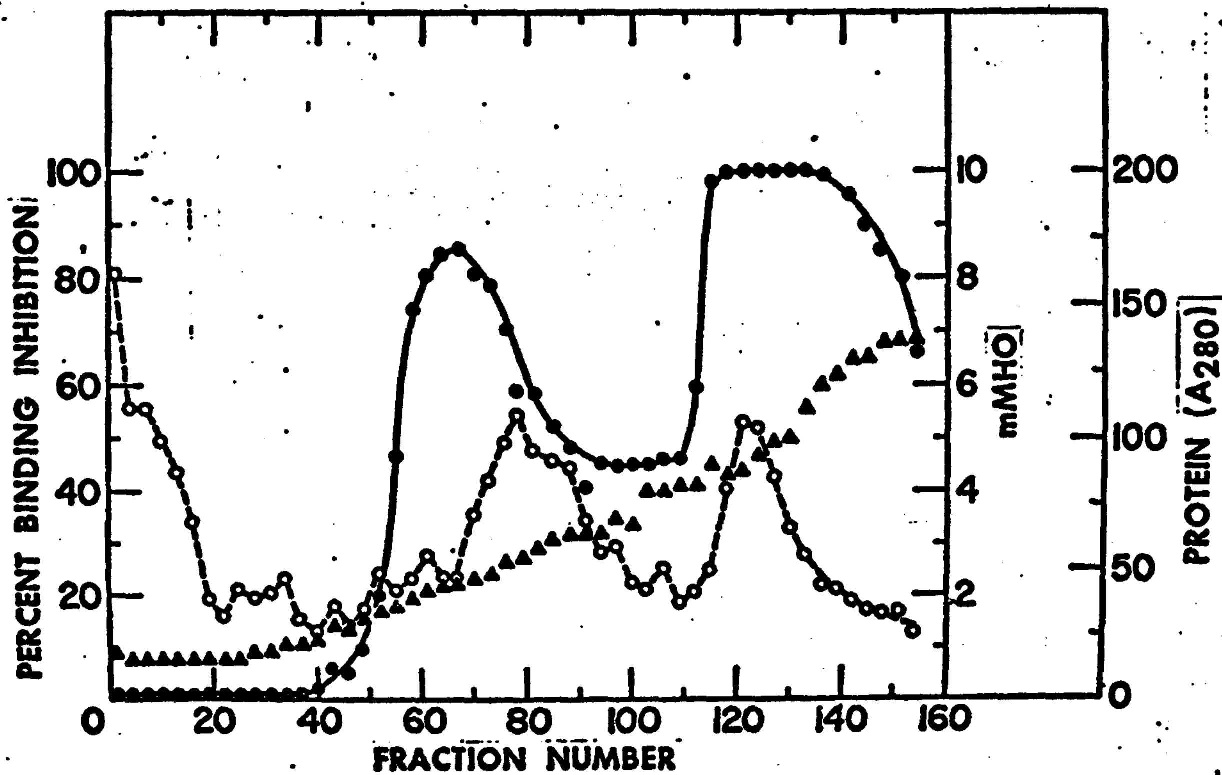


Figure 10. Elution profile from DEAE cellulose column. Legend as Figure 1, except that here the homologous radioimmunoassay was used, and protein were monitored by A_{280}.

by the modified preparation. The results were compared with those obtained for the mouse protein (82). The amino acid composition of both marker proteins were very similar (Table 3). In both cases Lys + Arg account for 13 mole percent and Glu + Asp for 30 mole percent of the amino acid assayed after performic acid oxidation. For all other amino acids, the content in both marker proteins is very similar. Molecular weight estimates, calculated from these amino acid analysis, were 20,000 daltons.

Amino acid analysis of the low molecular weight elution peak obtained from the Sephadex column was performed before and after hydrolysis. Before hydrolysis, the amino acid glycine was detected together with another unknown compound. After hydrolysis, 8 other unknown compounds were visible. Thus, the original peak eluted from the Sephadex column contained at least one peptide and the amino acid glycine.

Stability of the rat olfactory marker protein. During the purification of the rat olfactory marker protein, it became apparent that losses of immunological activity occurred. Thus, in some experiments after dialysis of an intermediate fraction (P_{95}) against a large volume of distilled water, only 30% of the immunological activity was recovered. A similar sample was assayed before and after dialysis against distilled water,against 0.01 M phosphate buffer pH 7.7, against either of these buffers containing 0.2 M NaCl or 10^{-3} M EDTA or dithiothreitol. No difference was visible.

High-speed supernatants and P_{95} fractions had comparable behaviors when dialysed, stored at 0-4°C, or at -20°C for 24 hours. There were losses in immunological activity ranging from 45% to 55% of the initial activities. Samples were kept for 22 days in those different conditions

TABLE III

	Rat Protein[1]		Mouse Protein[2]	
	Moles per 100	S.D.	Moles per 100	S.D.
Lys	8.03	0.19	7.8	0.2
His	0.68	0	0.89	0.06
Arg	5.4	0.24	5.4	0.28
Asp	13.4	0.53	12.6	0.28
Thr	5.0	0.15	5.0	0.14
Ser	3.8	0.16	5.45	0.49
Glu	16.3	0.4	17.1	0.28
Pro	4.94	0.15	4.55	0.02
Gly	5.85	0.14	6.95	0.92
Ala	6.68	0.02	7.02	0.53
Half Cys	--	0	--	0
Val	5.83	0.32	5.15	0.35
Met	2.46	0.05	1.9	0
Ile	3.15	0.05	3.27	0.10
Leu	12.9	0.15	11.6	0.56
Tyr	1.3	0.07	1.3	0
Phe	4.2	0.14	3.95	0.07
Trp[3]	4.6		1.3	
Lys + Arg	13.43		13.2	
Gly + Asp	29.7		29.7	

Amino acid analysis of purified rat and mouse olfactory marker protein. 1. Amino acid analysis was performed on the homogeneous sample obtained by the modified preparation and after 22 hrs hydrolysis in constant boiling HCl at 110°C. Results are mean of 3 experiments. 2. See Margolis (20) - Results are mean of 2 experiments. 3. Determined spectrophotometrically.

and assayed at days 1, 5, 6, 11, and 22. After the losses which occurred the first day, the immunological activity stayed constant, although for unknown reasons, one sample had sometimes higher immunological activity. Thus, the high speed supernatant always had 43% of the initial immunological reactivity except on day 6 when it had 60% of this initial activity. After identical times of storage, samples which had been kept frozen always had the highest immunological activity. Samples kept cold or dialysed had from 55 to 85% of the immunological activity of the identical sample kept frozen. Several aliquots of a high-speed supernatant were kept at 0-4°C in 0.01 M phosphate buffer pH 7.7 containing different compounds. Radioimmunoassays were performed at days 1, 3, 6, and 10. No difference was observed after addition of any of the following compounds at the indicated final concentration: 0.32 M sucrose, 0.2 M NaCl, 0.1% Triton, 50% glycerol, or after addition to a final concentration of 10^{-3} M of dithiothreitol, glutathione, pyridoxal phosphate, NAD, EDTA, cAMP, cGMP, carnosine, β-alanine, histidine, $ZnCl_2$, $MnCl_2$, $MgCl_2$.

Upon dialysis of the pure rat olfactory marker protein against distilled water or buffer, up to 70% of the immunological activity was lost by comparison to the identical sample kept cold.

Thermal denaturation of the pure rat olfactory marker protein was a complex phenomenon. The protein in solution in distilled water was first studied. The curve obtained on a semilogarithmic plot was never a straight line but a combination of several straight lines (Fig. 11). Denaturation at 37°C initially demonstrated a half life of 30 minutes. After 1 hr, the slope became less steep so that after 21 hrs, 12% of the immunological activity was still present. Total protein was monitored at

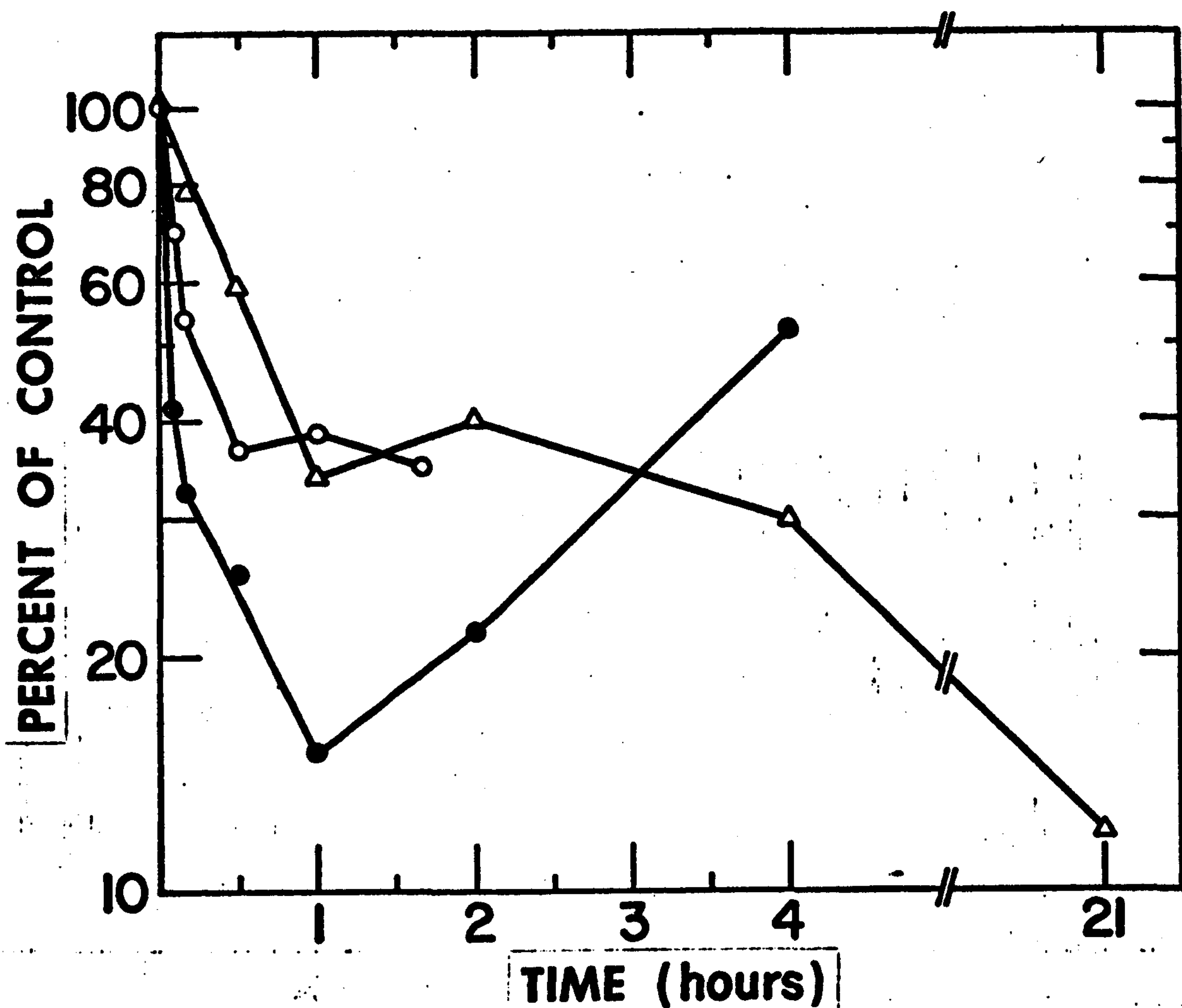


Figure 11. Thermal denaturation of pure rat olfactory marker protein. The homologous radioimmunoassay was used.

— • — • Protein in distilled water, at 100°C.

— o — o Protein in 50% glycerol, at 100°C.

— Δ — Δ Protein in distilled water, at 37°C.

100% corresponds to the immunological activity of one identical sample stored at 0-4°C during the experiment.

the same time and was constant during the incubations. Denaturation at 100°C in distilled water showed initially a half life of 5 minutes. The slope became less steep after 10 minutes and 15% of the immunological activity was recovered after 60 minutes incubation. Similar results were obtained with protein solutions in 0.01 M phosphate buffer pH 7.7 with or without 0.2 M NaCl.

The thermal treatment was also performed in presence of 50% glycerol. The initial half life was 30 min. After 1 hr, a plateau was reached at 35% of immunological reactivity (Fig. 11).

A reproducible property of the pure rat olfactory marker protein was its ability to recover immunological reactivity when incubated for longer than 1 hr at 100°C. Thus, after 4 hrs, the immunological reactivity was back from 15% to 45% of the initial value (Fig. 11).

DISCUSSION

The olfactory marker protein has now been isolated from rat olfactory bulbs and its physicochemical properties have been compared to those of the mouse marker protein. There were striking similarities between the olfactory marker proteins of the 2 species. Their molecular weights, as estimated by column chromatography or by SDS gel electrophoresis, are indistinguishable. Results of concanavalin A filtration show that the rat olfactory marker protein does not contain carbohydrates with a free α D-mannose or glucose (83) residue. This corroborates results obtained earlier with the pure mouse protein after gel electrophoresis (84) which provided no evidence for any carbohydrate by staining with periodic acid-Schiff reagent. The similarities between the 2 marker proteins is also manifest from the amino acid analyses. The amino acid compositions of these 2 proteins are nearly identical. No cysteine could be detected. The compositions are consistent with the fact that these are acidic brain proteins: the sum of the acidic amino acid residues (Glu + Asp) is more than twice the sum of the basic residues (Lys + Arg + His). However, in these amino acid analyses, we could not distinguish between glutamic acid and glutamine or aspartic acid and asparagine. Thus, there may be some differences in the 2 amino acid compositions in terms of amidation. This could account for some of the slight structural differences that we observed between the rat and the mouse olfactory marker protein. The pI for the mouse olfactory marker protein is 4.7 whereas the pI for the rat olfactory marker protein is 5.0. This probably explains the slightly faster mobility of the mouse protein observed in Ornstein-Davis polyacryla-

mide gels as compared to the rat protein.

The results of the molecular weight determinations of the olfactory marker proteins by different methods were puzzling. The molecular weight of the olfactory marker proteins estimated by polyacrylamide gel electrophoresis in the presence of SDS was 16,500 ± 1500 daltons. The results were identical when the proteins were preincubated with or without β-mercaptoethanol, and this is consistent with the absence of cysteine, as demonstrated by the amino acid analysis. This value was close to that of 18,000 found for the mouse protein by elution from a Sepharose 2B column in the presence of 6 M guanidine hydrochloride (84). The molecular weight of the native olfactory marker proteins estimated by column chromatography with Biogel P-60 and Sephadex G-75 was 30,000 daltons. These results suggested that the protein has a dimeric structure and that each monomer has a molecular weight of 15,000 daltons. Preliminary results from studies of the behavior of the protein by equilibrium sedimentation suggest that the protein may form a monomer-dimer system in equilibrium which is dependent upon ionic strength. Treatment with the cross-linking reagent dimethyl suberimidate hydrochloride did not give any indication of such a dimeric structure. However, this negative result does not eliminate the possibility of a dimeric structure of the protein since the cross-linking reaction will occur only if the ε amino groups of lysine are present in the appropriate conformation (78). Another possibility is that the pure protein forms aggregates. This is suggested by the comparison of Ornstein-Davis polyacrylamide gel electrophoresis (Fig. 9b) and SDS gel electrophoresis (Fig.4-4) of 10 μg of pure protein. Although a faint contaminating band is observed above the main protein band in the first

system, only one band is observed in the second system. Moreover, a small peak of immunologically active protein was eluted in the void volume of a Sephadex G-75 column (Fig. 8) and probably represents an aggregate of the pure protein. More work is required to solve the problem of the possible multimeric structure of the olfactory marker protein.

The molecular weight of the mouse olfactory marker protein, estimated by SDS gel electrophoresis, was not modified during preparation since labeled, newly synthesized protein in epithelium extracts co-migrated with purified mouse and marker protein (45). However, a difference in elution position between purified olfactory marker protein and native protein in fresh extracts was observed consistently for both mouse and rat by column chromatography on either Sephadex G-75 or on Biogel P-60. The native proteins, as present in the crude extract, had an apparent molecular weight of 30,000 daltons, whereas the purified proteins had an apparent molecular weight of 35,000 daltons. Tritium labeling by reductive alkylation of the pure protein did not modify this apparent molecular weight. Therefore, during the purification of the marker protein by both methods, a conformational change had occurred. We do not know what induces this change and whether it affects the immunological reactivity of the protein. The studies of the stability of the rat olfactory marker protein showed that upon storage, even at -20°C, some immunological activity of the protein was lost. In the presence of cofactors, metal ions, cyclic nucleotides, and all the other compounds tested, the same losses occurred. Thermal denaturation showed a complex behavior. Some protection of the immunologically active protein was achieved by 50% glycerol. More intriguing was the recovery of immunological activity after several hours at 100°C in dis-

tilled water. All this indicates that the conformation of the protein is modified. It is not known whether this protein had enzymatic activity nor whether the physiological properties of the protein are affected by these conformational modifications. Antisera produced by injecting the purified proteins cross-reacted with the native forms and were extremely specific (30, Chapter I). The difference in DEAE-cellulose elution pattern of the rat protein between the 2 purification procedures suggests that a structural change occurs during the purification of the rat olfactory marker protein. Whether this change is related to the change of apparent molecular weight between the native and purified olfactory marker protein and whether these conformational changes have any physiological significance are unknown. Further studies are needed to answer these questions.

The olfactory marker protein is synthesized in the olfactory epithelium (45) and presumably is transported by axoplasmic flow (45, 73) to the nerve endings in the olfactory bulbs. No structural differences could be detected in the marker protein between these 2 locations. The molecular weight of the mouse protein from the epithelium tissue extracts estimated by SDS gel electrophoresis was the same as that of protein purified from the olfactory bulbs (45). Molecular weight estimated by column chromatography was the same in both olfactory epithelium and olfactory bulb extracts from the rat and the mouse (Fig. 6, Fig. 7). Although some differences occur between the rat and the mouse proteins, the physicochemical studies of both olfactory marker proteins have shown striking similarities. The fact that immunologically similar (Chapter I) olfactory marker protein of a very distant species, the garfish, has the same apparent molecular weight as the mouse and the rat protein strongly suggests that some main

physicochemical features, presumably related to a function, have been conserved during evolution in all the proteins which cross-react with the rat olfactory marker protein. The evolutionary conservation of the olfactory marker protein has been strongly suggested by our previous immunological studies with the rat protein (Chapter I) which showed species cross-reactivity with olfactory tissue extracts from a wide range of vertebrates. We have now confirmed this by direct analysis of the chemical and physical properties of the mouse and the rat proteins and by preliminary observations with the garfish olfactory marker protein.

ACKNOWLEDGMENTS

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(III) SUBCELLULAR DISTRIBUTION OF BIOCHEMICAL MARKERS IN THE RAT OLFACTORY BULBS

The cell bodies of the olfactory chemoreceptor neurons are contained in the olfactory epithelium (Fig. 1). They extend ciliary processes peripherally to the surface of the epithelium (85). It is on the surface membrane of these processes that specific receptors are thought to bind odorant molecules (86). This is probably the site where the transduction mechanism occurs and transforms the binding of odorants to specific receptors into an action potential conveying the sensory information to higher centers. The marker protein is present in the cell bodies of the olfactory chemoreceptor neuron (45, 46, Chapter I), and, therefore, could possibly be implicated in the transduction mechanism. The axons of the olfactory chemoreceptor neurons extend their nerve terminals into the olfactory bulbs (35) where they synapse with the mitral cells in a specialized structure, the olfactory glomeruli. Studies of this primary olfactory synapse with the electron microscope show numerous synaptic vesicles and characteristic structures of chemical synapses (39). However, the putative neurotransmitter is not known. The marker protein is present in high concentration in the nerve terminals. More recently, Margolis has shown that carnosine is also a highly specific marker for the olfactory chemoreceptor neuron (47). It is thus possible that the function of the olfactory marker protein is related to the olfactory neurotransmitter or carnosine metabolism.

In order to investigate this, I decided to develop an *in vitro* system for metabolic studies of the olfactory marker protein, of putative neuro-

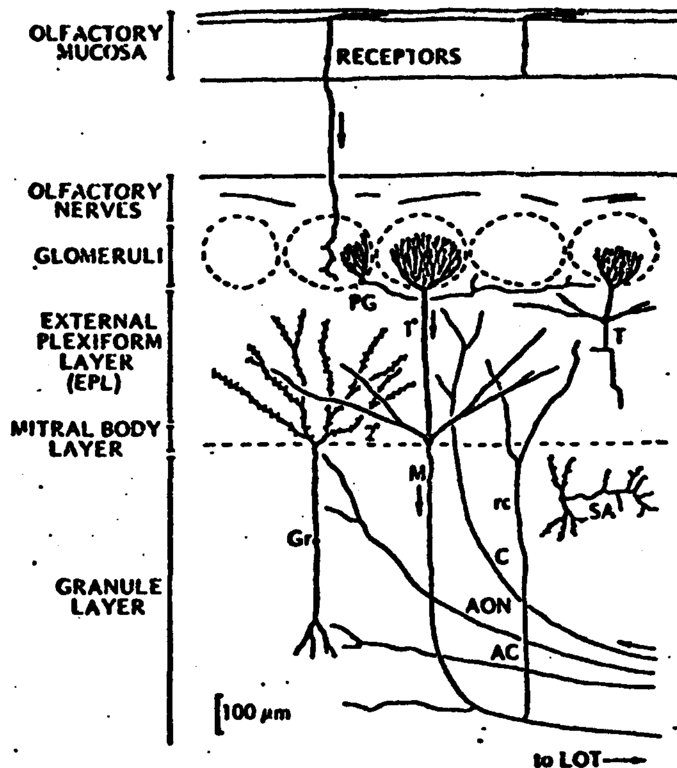

Figure 1. Structure of the mammalian olfactory bulb. (From Shepherd (35))

PG: periglomerular cell; T: tufted cell; M: mitral cell

SA: short axon cell; Gr: granule cell.

1° and 2° respectively are the primary and secondary dendrites of the mitral cell; rc: recurrent axon collateral.

The output is by the lateral olfactory tract (LOT). The central input is by the centrifugal fibers (c), fibers from the anterior olfactory nucleus (AON), and the anterior commissure (AC).

transmitters, and of the possible interactions between the marker protein and a putative neurotransmitter at that synapse. Such an in vitro system could be used to investigate the possible role of the dipeptide carnosine at the primary synapse of the olfactory nerve and whether it is related to the function of the olfactory marker protein.

As a result of extensive physiological and anatomical studies, the laminated structure of the olfactory bulb is one of the best understood of cortical systems (35). It has several distinctive features. It contains the synaptic glomeruli. A glomerulus represents a cluster of synapses. The input is formed by numerous olfactory nerve fibers, whereas the output consists of the dendrites of many fewer mitral and tufted cell dendrites (36). Moreover, the olfactory bulb is the first structure in which reciprocal dendrodendritic synapses have been described (87). They occur between the primary dendrites of the mitral cells and the dendrites of the periglomerular cells in the glomerular layer (88), as well as between the secondary dendrites of the mitral cells and the dendrites of the granule cells in the external plexiform layer. The granule cells are peculiar in that they do not have any axon on anatomical as well as on physiological analysis (35). The only output of the olfactory bulb towards cerebral centers is via the lateral olfactory tract which is formed of the axons of the mitral cells and of, at least, some tufted cells.

By contrast to the numerous anatomical and electrophysiological studies with the olfactory bulbs, very little biochemical or histochemical studies have been performed. Some studies have been concerned with the localization of putative neurotransmitters. Dahlstrom et al (89) have shown that norepinephrine fibers of central origin send nerve terminals to

the olfactory bulbs. They seem to synapse with granule cell dendrites in the granule layer and do not have any contact with the mitral cells. Their study also describes serotonin containing terminals in the external plexiform layer. Histochemical studies with acetylcholinesterase have been conducted (90) and showed that cholinergic fibers coming from the olfactory tubercle send outputs to the glomerular and mitral cell body layers. The existence of cholinergic fibers in the olfactory bulbs has been confirmed by another study (91) in which choline-acetyltransferase and acetylcholinesterase activities were evaluated. More studies have been concerned with the putative neurotransmitter of the granule cells. Electrophysiological and pharmacological studies (92, 93) have suggested that in the reciprocal dendrodendritic synapses between the mitral cells and the granule cells, the mitral to granule cell synapse is excitatory. From iontophoretic studies (94), norepinephrine, acetylcholine, as well as excitatory amino acids, could be the putative neurotransmitter. The granule to mitral cell synapse is inhibitory. Iontophoretic studies with GABA, glycine, β-alanine, and the effect of the GABA inhibitors bicuculline, and picrotoxin suggest that GABA is the putative neurotransmitter. This hypothesis has been further reinforced by histochemical studies (95). High glutamic acid decarboxylase activity, as well as high GABA levels, were found in the external plexiform layer where this dendrodendritic synapse occurs. Glutamic acid decarboxylase and GABA were also high in the glomerular layer suggesting that the reciprocal dendrodendritic synapses between periglomerular cells and mitral cells may be comparable to the synapses between mitral and granule cells.

Thus, the known specific biochemical markers in the olfactory bulbs

are: GABA and glutamic acid decarboxylase in the granule cells and the olfactory marker protein, carnosine and carnosine synthetase for the olfactory chemoreceptor fibers. Moreover, cholinergic, noradrenergic, and serotonergic fibers have also been described.

Subcellular fractionation techniques were first applied to the nervous system in the 1950's by both the Whittaker (96) and de Robertis groups (97) using the cerebral cortex of rats or guinea pigs. After homogenization of the tissue in an isotonic medium (0.32 M sucrose) the homogenate was submitted to differential centrifugation,and the different fractions were analyzed by electron microscopy as well as by the use of biochemical markers. The remarkable property of the nerve endings to "pinch off" during homogenization and to form organelles was thus observed. These organelles were called synaptosomes by Whittaker. They contain cytoplasm, mitochondria, and the synaptic vesicles characteristic of the nerve endings. Very often pieces of postsynaptic membrane are still attached to the presynaptic membrane of the synaptosomes. The synaptosomes, formed during the homogenization of the cerebral cortex, sediment with free mitochondria but they can be separated in a purer fraction on a sucrose gradient. When submitted to osmotic shock, the synaptosomes will release the synaptic vesicles which may be further purified on another density gradient (98).

In vitro preparations of synaptosomes and of vesicles have been widely used and have been greatly helpful to our understanding of synaptic mechanisms. However, most of this work has been concerned with cerebral cortex. In some early attempts,subcellular fractionation of the cerebellum was carried out, and the complex nerve endings (glomeruli) of the mossy

fiber neurons were shown to sediment in a fraction different from the classical synaptosomal fraction (99). These giant synaptosomes sedimented at a lower speed with denser particles such as nuclei and cell debris. Recently, Balazs' group has succeeded in isolating a purified fraction of these glomeruli from the rat cerebellum (100). The structure of the olfactory glomeruli, like that of the cerebellar glomeruli, is a complex multisynaptic structure. However, in the cerebellum the transmitter involved in the nerve terminals of the mossy fiber neuron of the glomerulus is not known and no specific biochemical markers have been described. Therefore, these workers mainly used electron microscope observations to ascertain the purity of their fraction. In the case of the olfactory bulbs, we benefit from our knowledge of the existence of specific biochemical markers of the olfactory chemoreceptor neuron. I, therefore, decided to attempt to isolate the macrostructure formed by the glomeruli in the olfactory bulb and to follow this subcellular fractionation procedure by morphological and biochemical techniques.

MATERIALS AND METHODS

Male Sprague-Dawley rats (5-6 weeks old) were sacrificed by decapitation , and the olfactory bulbs were quickly removed and chilled on ice. Ten to twenty animals were killed for each experiment. All the following procedures were at 0-4°C.

Subcellular fractionation: We used a procedure similar to that described for the isolation of cerebellar glomeruli (100). The tissue (1-2 g) was manually homogenized with a Dounce homogenizer in 9 volumes of 0.32 M sucrose containing 1 mM $MgSO_4$. The tissue was disrupted by using a pestle of 0.12 mm clearance and 50 up and down strokes. In order to remove capillaries and cell debris, the homogenate was filtered under gentle pressure through a series of nylon filters of 1000, 200, and 70 μm pore size. Approximately 20% of the original homogenate content of total protein and of each of the biochemical markers was lost at this stage. The filtered homogenate (H) was then submitted to differential centrifugation. At each step of the fractionation, 1 ml aliquot was kept. The filtered homogenate was centrifuged for 10 min at 1000 g in a Sorval centrifuge and yielded a pellet (P_1) containing the nuclei, axon, and presumed glomeruli fragments. The supernatants (S_1) was further fractionated by centrifuging for 20 min at 14,500 g. The crude mitochondria pellet (P_2) and the supernatant (S_2) were then recovered. In an attempt to obtain a purer glomeruli preparation, the pellet (P_1) was further fractionated on a continuous sucrose gradient. All the sucrose solutions contained 1 mM $MgSO_4$. A continuous sucrose gradient (0.32 M - 1.4 M) was formed on a cushion of 1.6 M sucrose and kept overnight at 40°C. A

freshly prepared P_1 fraction was layered on the gradient. The gradient was centrifuged at 53,000 g for 2 hrs in a Spinco ultracentrifuge using the SW25.1 rotor. The gradient was collected from the top in 1 ml fractions by displacement with 2 M sucrose.

Determination of biochemical markers: Proteins were assayed by the fluorescamine method, using BSA as a standard (52).

The olfactory marker protein was determined by the homologous radioimmunoassay (Chapter I). Aliquots for radioimmunoassay were adjusted to 0.2% SDS. After 10 min at room temperature, they were diluted with 10 volumes of cold distilled water, and 100 µl of the final solution was used for the radioimmunoassay.

Lactate dehydrogenase (LDH) was used as a non specific soluble cytoplasmic marker and was assayed spectrophotometrically (101). An aliquot (usually 100 µl) was adjusted to 1% triton-X 100 and was incubated for 10 min at room temperature. Then, 0.05 M Tris HCl buffer pH 7.4 containing 0.1 mM NADH and 1.2 mM KCN was added to a final volume of 3 ml. When the absorbance at 340 mµ stabilized (3-5 min), pyruvate (0.5 mM final concentration) was added, and the linear decrease in the absorbance of 340 mµ was recorded for about 5 min.

Fumarase was chosen as the mitochondrial marker and was also assayed spectrophotometrically (102) after pretreating the aliquots with 1% triton-X 100 at room temperature. To the sample (usually 100 µl), 3 ml of 0.05 M NaH_2PO_4/Na_2HPO_4 buffer pH 7.4 containing 34 mM malic acid were added. The formation of the double bond was observed by monitoring the increase in the absorbance at 250 mµ during 5 min.

5'-Nucleotidase was a plasma membrane marker (103) and was quantitated

by determination of the inorganic phosphate released from 5' AMP at 37°C (104). The sample was first treated with 0.25% triton for 30 min in the cold then added to cold 75 mM Tris pH 9.0 containing 10 mM KCl, 5 mM $MgCl_2$ and 5 mM 5' AMP and incubated for 1 hr at 37°C. The reaction was stopped by the addition of 10% TCA. After centrifugation at 1000 g for 5 min, inorganic phosphate in the supernatant was measured.

Choline acetyltransferase is a marker for the cholinergic neurons and was assayed by the method described by Wilson et al (105). The samples were first treated for 30 min with 50 mM K_2HPO_4/KH_2PO_4 pH 6.8 buffer containing 200 mM NaCl and 0.5% triton in the cold. After incubation for 10 min at 37°C in presence of 1 mM choline chloride and 0.2 mM [1-^{14}C] acetyl Co-A, the reaction was stopped by addition of 1.5 ml of cold distilled water. [^{14}C] acetylcholine resulting from the enzyme catalyzed reaction of [^{14}C] acetyl-CoA with choline acetyltransferase was not retarded by a column (3 x 0.5 cm) of Dowex AG-1 and was collected into scintillation vials and counted in the presence of Aquasol.

Glutamic acid decarboxylase is a marker for the GABA containing neurons. The activity was estimated by measuring the release of [^{14}C] CO_2 from [1-^{14}C] glutamic acid (102). The samples were kept 30 min on ice in presence of 0.25% triton and 0.05 M potassium phosphate pH 7.0. They were then added to the incubation medium which contained 1 mM aminoethylisothiouronium bromide, 0.1 mM pyridoxal phosphate, and 50 mM [1-^{14}C] glutamic acid and had been gassed with N_2 prior to the addition of the sample. The activity thus measured was inhibited by 10^{-3} M aminooxyacetic acid. All these enzymatic assays were performed in duplicate.

The particulate subcellular fractions were characterized regarding

their uptake properties for GABA (γ-amino butyric acid), histidine, β-alanine, carnosine, glutamic acid, and choline. The fractions were incubated in the medium described by Bradford (106) after pre-equilibration with 95% O_2: 5% CO_2. The composition of the medium was the following (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.2; $MgSO_4$, 1.3; $CaCl_2$, 0.75; $NaHCO_3$, 26; glucose, 5; pH 7.4. In some experiments, NaCl, 124 mM was replaced by sucrose 248 mM. An aliquot of the P_1 or P_2 fraction containing 0.3-0.5 mg protein was added to 2 ml of medium and preincubated 5 min at 37°C. The uptake was initiated by adding 20 μl of the labeled compound in the appropriate concentration to monitor a high affinity uptake system. The reaction was stopped after 5 min by immediate suction filtration through a millipore filter (0.45 μM pore size) followed by a wash with 5 ml of the incubation buffer at room temperature (107). The filter pads carrying the tissue were transferred tp scintillation vials containing 3 ml of water, and after 1 hr at room temperature, 10 ml of Aquasol was added and the radioactivity in the samples determined in a scintillation counter. Blank values were determined by incubating identical fractions in the presence of the labeled compound, but at 0°C, and washing the pads with cold incubation medium. In one study, comparable conditions and amount of tissue were used but cortex slices instead of the subcellular fractions P_1 and P_2 were tested. The slices were prepared as described by Margolis <u>et al</u> (46). The cortex of 1 animal was cut on a McIlwain tissue chopper (Brinkman) into 0.1 mm slices and then cut a second time at the 0.1 mm setting at a right angle to the direction of the first cut. The chopped tissue was suspended to give a final concentration of 2 mg weight/ml incubation medium. Every uptake was performed in triplicate. In each experi-

ment, GABA uptake was used as a control.

Electron microscopy: The method described by Cotman and Flansburg (108) was used. A drop of sample was added to 1 ml of 1% O_sO_4 in 0.1 M phosphate buffer, pH 7.4 containing a final concentration of 0.32 M sucrose and allowed to stand for fixation during at least 30 min. This allowed the formation of a 1 mm thick pellet after centrifugation (20,000 g for 10 min) which could be treated as 1 piece of tissue permitting a survey of the full thickness of the fixed pellet. The pellet was dehydrated in a graded acetone series followed by anhydrous propylene oxide. The dehydrated specimens were embedded in Epon 812 and sectioned. The thin sections were stained with 2% uranyl acetate solution (ethanol-water 2:1), washed, and contrasted with 0.4% lead citrate solution in 0.1 M sodium hydroxide. The grids were stabilized by deposition of very thin carbon film and examined in a JEM-100B electron microscopy at 80 KV.

[2,3-^{3}H] γ-aminobutyric acid (10 Ci/mmole), [3-^{3}H] glutamic acid (2.8 Ci/mmole), [3-^{3}H] β-alanine (37.5 Ci/mmole), [1-^{14}C] acetyl CoA (54.7 mCi/mmole), [1-^{14}C] β-alanine (28.7 mCi/mmole were purchased from New England Nuclear. [Methyl-^{3}H] choline chloride (10 Ci/mmole), [D-L, 1-^{14}C] glutamic acid (20 mCi/mmole), [2,5-^{3}H] histidine (55 Ci/mole) were purchased from Amersham Searle. [^{14}C] carnosine (11.2 mCi/mole) was purchased from Calatomic and ^{3}H carnosine (57.6 μCi/μmole) was synthesized by Hoffmann LaRoche (RO14361). All other materials were reagent grade.

RESULTS

Distribution of the biochemical markers in the subcellular fractions: The recoveries of each marker in the different subcellular fractions are reported in Table I. They show a marked difference between the subcellular distribution of the rat olfactory marker protein and the distribution of the other specific biochemical markers, choline acetyltransferase and glutamic acid decarboxylase. A high proportion of the olfactory marker protein (about 40%) was recovered in the soluble fraction S_2. The only other soluble marker recovered in S_2 with a high percentage was LDH, the non specific cytoplasmic marker, with 52% of activity in this fraction. The soluble markers specific for acetylcholine and GABA containing neurons were recovered at a much lower percent in that soluble fraction, 13% for choline acetyltransferase and 24% for glutamic acid decarboxylase. A very low recovery was obtained for fumarase, the mitochondrial marker, but 19% of the plasma membrane marker 5' nucleotidase was present in this fraction probably on microsomes. A lower proportion of rat olfactory marker protein was recovered in the particulate fractions, P_1 and P_2 together, than of the other specific soluble markers choline acetyltransferase and glutamic acid decarboxylase. About 25% of the total proteins sedimented with the low speed pellet P_1. The enzyme markers associated with the mitochondria and the plasma membranes, fumarase and 5' nucleotidase respectively, sedimented in a slightly higher proportion (30 and 32%) whereas a lower percent of the soluble markers LDH, choline acetyltransferase, and glutamic acid decarboxylase (19 to 23%) sedimented with the P_1. The proportion of marker protein sedimenting with P_1 was intermediary and 27%. In the mitochondrial pellet P_2, 45% of the total proteins were recovered. This value is inter-

TABLE I

PERCENTAGE DISTRIBUTION OF BIOCHEMICAL MARKERS IN SUBFRACTIONS OF RAT OLFACTORY BULBS

	n	Specific Activity in H	% Recovery of H			
			P_1	S_1	P_2	S_2
Rat olfactory marker protein	6	730 ± 196	27.9 ± 9	68 ± 13	23.4 ± 3.5	38 ± 6
Lactic dehydrogenase	5	110 ± 31	20.5 ± 4.5	96 ± 19	32 ± 5	52 ± 6
Glutamic acid decarboxylase	4	211 ± 60	23 ± 6	67 ± 3	42 ± 9	24 ± 2
Choline acetyl transferase	3	635 ± 95	19 ± 4	83 ± 34	54 ± 16	13.5 ± 2
Fumarase	3	6.5 ± 0.17	30.6 ± 2	68 ± 9	48 ± 18	9.3 ± 0.4
5'-nucleotidase	2	1.00 ± 0.08	32.5 ± 2	70.5 ± 0.7	40.5 ± 10	18.9 ± 1.2
Protein	7		25 ± 5	67.7 ± 8	45 ± 11	16 ± 3

n = number of experiments where the determination was made.
In each experiment, the assays were performed in duplicate. Units: rat olfactory marker protein: ng, lactic dehydrogenase and fumarase: ΔOD 10^{-2}/mn, glutamic acid decarboxylase: nM CO_2/hr, choline acetyltransferase: pMole Ach/min, 5'-nucleotidase; μM Pi/hr. Specific activity was expressed as unit/μg protein.

mediary between those obtained for the particulate markers, fumarase and 5' nucleotidase, and the specific soluble markers glutamic acid decarboxylase and choline acetyltransferase, which are present in this fraction with a higher percentage than in P_1. However, only 32% of the LDH activity and 23% of the olfactory marker protein were recovered in the P_2 fraction. Thus, 50-60% of the particulate rat olfactory marker protein is contained in P_1 whereas about 40% of the particulate LDH and only 30-40% of the particulate glutamic acid decarboxylase and 20-30% of the particulate choline acetyltransferase are recovered in this fraction. This difference in the subcellular distribution of the olfactory marker protein and the distribution of the other soluble markers is seen as well as on Table II which shows the relative specific activities (RSA) of each biochemical marker. The RSA is calculated as follows: $RSA = \frac{\text{percent recovery of one marker}}{\text{percent recovery of total protein}}$ and, therefore, indicates the change in specific activity of one marker in one fraction by comparison to its specific activity in the initial homogenate. Whereas the P_2 fraction (RSA = 1.20) is enriched in choline acetyltransferase by comparison to P_1 (RSA = 0.75), the olfactory marker protein has a very low RSA in this fraction (0.52), lower than in P_1 (1.09), and also lower than the relative specific activity of any other markers. This reflects the enrichment of the marker protein in the P_1 fraction.

Distribution of the olfactory marker protein on a continuous sucrose gradient: After centrifugation of the P_1 fraction in the continuous sucrose gradient, 2 main bands were visible (Fig. 2). A large white band was closer to the top. The band closer to the bottom was less transparent, brown and white, and seemed to contain more material. Just under this large band, a narrow band containing red material was visible. A small red pellet was also visible. The distribution of the olfactory marker protein

TABLE II

RELATIVE SPECIFIC ACTIVITIES OF BIOCHEMICAL MARKERS

IN SUBFRACTIONS OF RAT OLFACTORY BULBS

Marker	P_1	S_1	P_2	S_2
Rat olfactory marker protein	1.09	1.00	0.52	2.33
Lactic dehydrogenase	0.8	1.42	0.71	3.19
Glutamic acid decarboxylase	0.90	0.99	0.93	1.47
Choline acetyl transferase	0.75	1.22	1.20	0.83
Fumarase	1.20	1.00	1.06	0.57
5'-nucleotidase	1.28	1.04	0.90	1.16

The relative specific activities (RSA) were calculated using the data presented in Table I. For each biochemical marker RSA =

$$\frac{\text{\% recovery of the marker in the fraction}}{\text{\% recovery of total protein in the fraction.}}$$

was compared to the distribution of total protein and of choline acetyltransferase (Fig. 3). The total protein distribution was in 3 peaks. The peak A closer to the top contained 6% of the total proteins, 15% of the olfactory marker protein, and 9% of the choline acetyltransferase. The peak C closer to the bottom contained 65% total proteins and the highest amount of olfactory marker protein (80%) and of choline acetyltransferase (80%). The middle peak B contained 19% of total proteins, did not contain any detectable olfactory marker protein, and very low choline acetyltransferase (9% of total activity). The pellet contained 10% of total proteins, 4% of marker protein, and 6% of choline acetyltransferase. When compared to the distribution of the bands visible on the gradient (Fig. 2), this middle peak B corresponds to the wide white band close to the top,and the heavier peak C corresponds to the heavier band. The peak A represents soluble material which has leaked from the particulate fraction during the procedure. The specific activities of rat olfactory marker protein and choline acetyltransferase are represented in Fig. 4.

For both markers, the specific activities obtained in these peaks are higher than the specific activities in P_1. Moreover, the heavier peak C did not show a distribution of the specific activities comparable to the distribution of total activities but was split into 2 peaks. This was reproducible and occurred for both the rat olfactory marker protein and the choline acetyltransferase. The specific activity of rat olfactory marker protein attained in the peak A of soluble proteins was 2.53 ng/μg. much higher than the specific activity in P_1 (0.93 ng/μg) and comparable to the specific activity in S_2 (2.22 ng/μg). The 2 heavier peaks of olfactory marker protein specific activities reached 1.76 and 1.60 ng/μg.

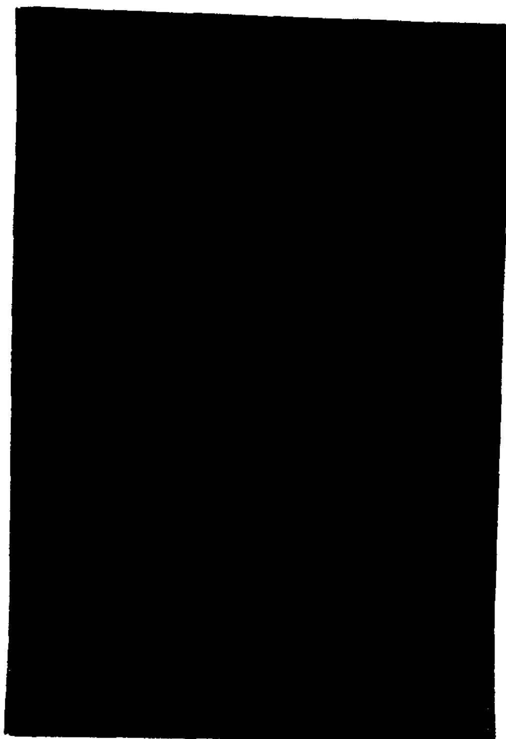

Figure 2. Fractionation of P_1 on a continuous sucrose gradient.

The sucrose gradient was formed with 1.4 M sucrose and 0.32 M sucrose on a cushion of 1.6 M sucrose. After loading 2 ml P_1 on top of the gradient, centrifugation was run for 2 hrs at 53,000 g in a SW 25.1 rotor.

Figure 3. Distribution of total proteins, rat olfactory marker protein and choline acetyltransferase on a continuous sucrose gradient.

▲ ▲ ▲ sucrose gradient; ●-●-● rat olfactory marker protein estimated by the homologous radioimmunoassay (ng/ml); -o-o-o- total protein (μg/ml); -Δ-Δ-Δ- choline acetyltransferase (pM/min x ml).

Figure 4. Specific activity distribution of the rat olfactory marker protein and choline acetyltransferase on a continuous sucrose gradient.

▲ ▲ ▲ sucrose gradient; -●-●-●- rat olfactory marker protein; -o-o-o- choline acetyltransferase. The specific activity is expressed as unit/μg protein.

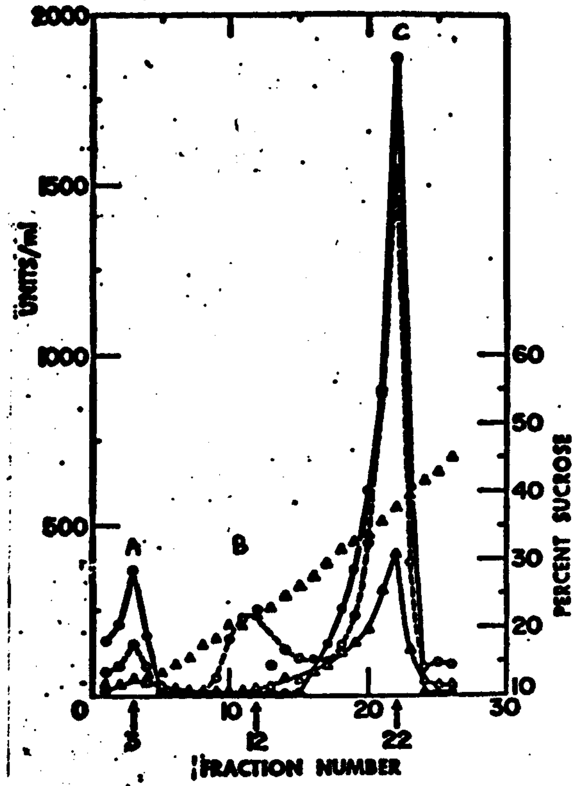

Figure 3

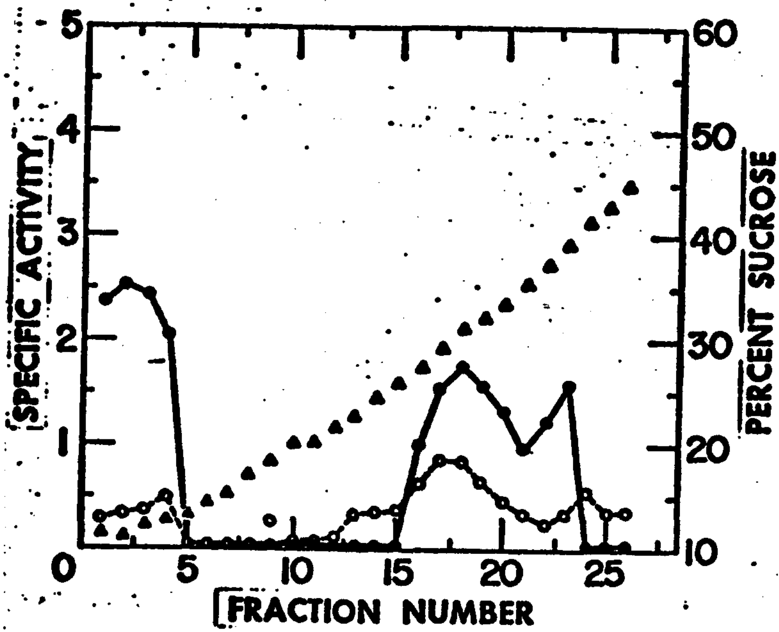

Figure 4

A similar repartition was obtained with choline acetyltransferase although the peaks of specific activities did not coincide with those obtained for the olfactory marker protein. The highest specific activities of choline acetyltransferase obtained on the gradient were 0.50 units/μg in the soluble peak A, 0.89 units/μg, and 0.53 units/μg in the heavier double peak C were clearly above the values obtained for P_1 (0.25 units/μg) and for S_2 (0.41 units/μg).

Uptake studies: The P_1 and P_2 fractions were further characterized by their ability to take up GABA, glutamic acid, choline, carnosine, β-alanine, and histidine by a high affinity mechanism. The results are shown in Table III. A high affinity uptake mechanism was observed for all the compounds tested except carnosine. All the compounds tested were tritium labeled. In the case of carnosine and β-alanine, the experiments were repeated with the ^{14}C-labeled compounds and gave identical results. Only GABA and choline were taken up preferentially by the P_2 fraction. These uptakes were temperature dependent. Furthermore, as shown in Table IV for GABA, this uptake was Na+ dependent, and there was only a slight inhibition of GABA uptake in the presence of a high concentration of β-alanine. We could not show any high affinity uptake of ^3H or ^{14}C-labeled carnosine in P_1 or P_2. No high affinity uptake of ^3H carnosine was observed in cortex slices.

Electron microscopy: The P_2 pellet was very rich in mitochondria and in small synaptosomes (Fig. 5). Different kind of synaptosomes could be seen, some being richer in vesicles than others. In some cases, post-synaptic membranes were still attached to the synaptosomes. In both P_1 (Fig. 6) and P_2, large myelinated structures were seen which presumably

TABLE III

UPTAKE STUDIES WITH THE PARTICULATE SUBFRACTIONS OF THE RAT OLFACTORY BULBS

Substrate	Final Concentration in medium	Number of Experiments	Uptake (pmoles/mg protein) P_1	P_2	$\frac{P_2}{P_1}$
GABA	5 μM	4	720 ± 193	1180 ± 190	1.7 ± .4
Histidine	5 μM	3	117 ± 30	152 ± 80	1.24 ± .34
Choline	0.05 μM	2	3.5 ± 1.3	5.2 ± .7	1.6 ± .4
β-Alanine	5 μM	2	26.4 ± 1.8	30.2 ± 5.9	1.15 ± .15
Carnosine	8.8 μM	4	0	0	
Glutamic acid	5 μM	2	612 ± 183	736 ± 258	1.19 ± .07

All results are expressed as mean of all the determinations ± standard deviation. In each experiment, the determinations were performed in triplicate. GABA uptake was routinely monitored as a positive control in every experiment.

TABLE IV

GABA UPTAKE IN THE PARTICULATE SUBFRACTIONS OF RAT OLFACTORY BULBS

Substrate	Final Concentration in Medium	Uptake in the Fraction (pmoles/mg Proteins)				
		P_1	% Inhibition	P_2	% Inhibition	$\frac{P_2}{P_1}$
GABA	5 µM	726.5		956.6		1.4
GABA ($-Na^+$)*	5 µM	88.8	88	132.8	86	1.5
GABA	5 µM	628.4		1260.4		2.0
GABA + β-Alanine (100 µm)	5 µM	560.6	11	926.7	26.5	1.6

All determination were performed in triplicate.

*In this case, NaCl (124 mM) was replaced by sucrose (248 mM).

Figure 5. P_2 fraction or crude mitochondrial pellet.

This fraction was obtained by centrifugation of S_1 for 20 min at 14,500 g. The horizontal bar at the bottom of the picture indicates 1 μ. The arrows point to the synaptic sites where the thickening of the membrane is often visible. Synaptosomes (S) contain mitochondria (M) and synaptic vesicles. The postsynaptic membrane is often still attached to them. Larger mitochondria, not contained in synaptosomes, are also visible as well as fragments of myelinated axons (A). x 14,300.

Figure 6. P_1 fraction.

This fraction was obtained by centrifugation of the filtered homogenate H for 10 min at 1000 g.

The legend is as in Figure 5. However, this fraction also contains nuclear material (N). Structures resembling synaptosomes are clustered together in synaptic complexes (C). Structures containing denser material and synaptic vesicles presumably are nerve endings of the primary olfactory fibers.

Figure 5

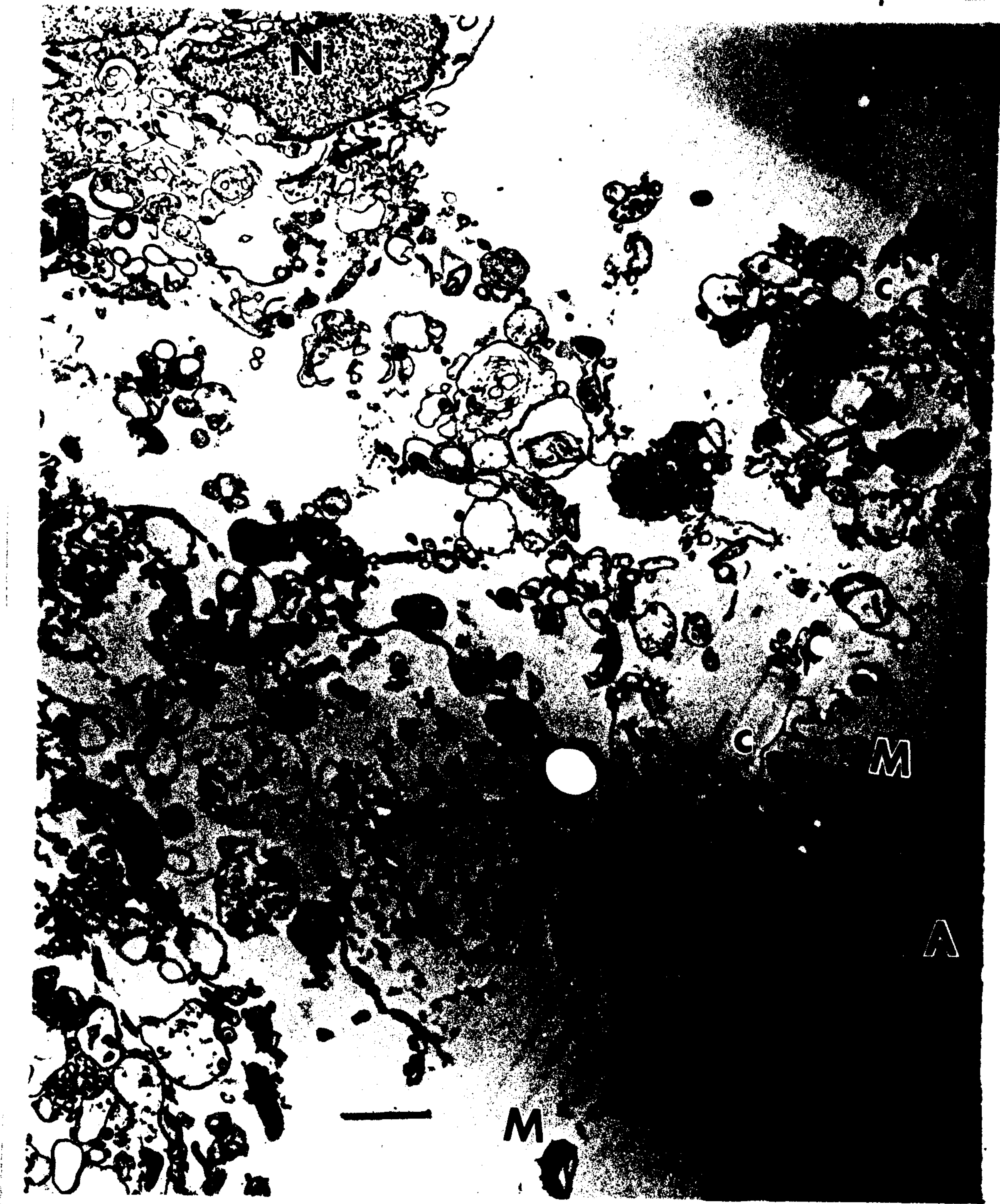

Figure 6

are pieces of axons. The P_1 fraction contained nuclear material and larger mitochondria than were seen in the P_2. Furthermore, in P_1, structures which are similar to synaptosomes, were observed. However, these structures are more complex since frequently we could see as many as 4 synaptosomes still attached together. These synaptic complexes contain some synaptosomes which are filled with very dense material and vesicles. This has been previously described for the olfactory nerve fibers (42). These synaptic complexes are presumably fragments of the olfactory glomeruli.

DISCUSSION

During the subcellular fractionation, a portion of the olfactory marker protein is associated with a particulate fraction which sediments in a different manner than the particulate material containing the other soluble markers, lactic dehydrogenase, choline acetyltransferase, and glutamic acid decarboxylase. The low speed pellet P_1, in which I expected to recover the glomeruli fragments, is enriched in particulate associated olfactory marker protein with a relative specific activity twice that observed in the P_2 fraction. The highest relative specific activity is attained in the soluble fraction S_2. Thus, the rat olfactory marker protein behaves in S_2 very much like a soluble protein and like the cytoplasmic biochemical marker LDH. In contrast to the other soluble markers, a large amount of the olfactory marker protein which gets occluded during the homogenization is trapped in larger particles which sediment in the P_1 fraction. The crude mitochondrial pellet P_2 which contains most of the synaptosomes is enriched in choline acetyltransferase and preferentially takes up choline by a high affinity uptake mechanism. This is in agreement with the fact that cholinergic fibers coming from central pathways synapse in the olfactory bulbs, presumably outside of the glomerular layer (90). Although there is no enrichment in glutamic acid decarboxylase activity visible in the P_2 fraction, the high affinity uptake for GABA is more efficient in this fraction than in the P_1 fraction. It is generally accepted that the high affinity uptake is a property of the nerve endings (109). Thus, the difference in the subcellular distribution of glutamic acid decarboxylase activity and of GABA high affinity uptake suggests that the P_2 fraction is enriched in GABA containing nerve termi-

nals. Axon fragments containing the soluble glutamic acid decarboxylase, are very probably present in P_1 and do not take up GABA. The electron micrographs (Fig. 5 & 6) show that the P_1 fraction is more heterogeneous than the P_2 fraction and, therefore, support this hypothesis. Figure 6 shows that the P_1 fraction is very heterogeneous containing nuclear material, pieces of axons, and synaptic complexes which presumably are olfactory glomeruli fragments, bigger synaptosomes, and mitochondria. Thus, we cannot distinguish between glutamic acid decarboxylase contained in axon fragments, in synaptosomes, or in glomeruli. The same is true regarding the subcellular localization of the other soluble markers in the P_1 fraction.

It has been shown that glial cells may also take up putative neurotransmitters or neurotransmitter precursors by temperature and sodium-dependent high-affinity uptake mechanisms (110). However, in the case of GABA, it was demonstrated that these 2 high-affinity uptake mechanisms could be distinguished (111). The β-alanine competes for the GABA uptake in glial cells but not in neuronal cells. The observation that 100 mM β-alanine inhibited the GABA high-affinity uptake only by 10% suggests that the observed GABA uptake is principally due to neuronal uptake.

The P_1 fraction is enriched in particulate material containing the rat olfactory marker protein. But it is not clear whether this particulate material is composed uniquely of the olfactory glomeruli or also of axon fragments derived from the olfactory nerve. I attempted to further characterize this P_1 fraction regarding the uptake of carnosine and of its precursors β-alanine and histidine. However, no difference could be observed between P_1 and P_2. The observation that carnosine could not be

concentrated by these fractions nor by cortex slices by a high affinity uptake mechanism, in conditions where we could observe the high affinity uptake for GABA was in contrast to a previous report. In this report (112), carnosine uptake into cortex slices was studied. A tissue to medium ratio of 6 was observed after 1 hr, and a ratio of 12 after 2 hrs. We did not observe any uptake significantly higher at 37°C than at 0°C, even after 2 hrs incubation. However, other authors (113) have observed a much less dramatic uptake of carnosine in cortex slices. They report a tissue to medium ratio of 2.5 after 1 hr and of 3 after 2 hrs.

In order to obtain a purer glomeruli fraction, a further fractionation of P_1 was attempted by a continuous sucrose gradient centrifugation. Only a small amount of the soluble markers (peak A) were released during the procedure, showing the relative stability of the subcellular structures. The high specific activities attained for both the olfactory marker protein and choline acetyltransferase in the heavy peak (C) of particulate material indicate that this material is purer than the P_1 fraction. Contaminating structures have been eliminated in the lighter band visible on the gradient (peak B). It would be interesting to identify this material, to investigate its uptake properties, whether it is formed of myelin, and whether it contains other neurotransmitter enzymes than those assayed in our experiments. The particles containing the rat olfactory marker protein and choline acetyltransferase are not separated on this gradient. However, there is a reproducible difference in distribution when the specific activities rather than the total activities are plotted. This suggests that it may be possible to separate particles containing the rat olfactory marker protein from those containing the choline acetyltransferase. A better separation

could possibly be achieved by using a ficoll sucrose gradient (114). In this case, the fragments would not be submitted to hyperosmotic shock and would better conserve their integrity and differences. Another possibility is to still use a sucrose gradient but only with a short centrifugation time (115). In this case, the particles are separated not as a function of their density but of their velocity in the sucrose gradient. Further separation of the particles containing the rat olfactory marker protein may yield a relatively pure preparation of the glomeruli by one of these methods. Studies with biochemical markers, analysis of the uptake properties, and observations with the electron microscope should confirm this. Such a preparation would be very useful for further metabolic studies of the olfactory glomeruli. However, even with a crude preparation like P_1, at least preliminary studies could be performed. It should be very interesting to study the precise location of the rat olfactory marker protein in this crude preparation by an immunohistological technique. Autoradiographic studies would show in which subcellular particles of P_1, GABA, β-alanine, histidine, choline are taken up. Furthermore, it would be interesting to investigate whether depolarization with high concentration of potassium releases specifically carnosine or the marker protein or both. Another approach concerning the role of carnosine would be to investigate whether such a preparation is enriched in binding sites for carnosine. These experiments could indicate whether carnosine is a putative neurotransmitter at the primary olfactory synapse. Determinations of carnosine levels in subcellular fractions await the development of a more sensitive carnosine assay.

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