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Studies on Serotonin Binding Proteins of Nerve Ending Membranes

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With 6 Figures

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Summary

Synaptic membranes were isolated from rat brain homogenates by differential and density gradient centrifugation. Membrane proteins were solubilized by detergent buffer and assayed for serotonin-binding activity by adsorption of free 5-HT on charcoal. When the membrane extract was incubated with serotonin at +4 °C for various times, equilibrium was reached within 10 min. With increasing serotonin concentrations the specific part of binding was saturable whereas the non-specific part increased linear with the total 5-HT added. Kinetic analysis of the data revealed two different classes of binding sites with the apparent dissociation constants $K_{d1} = 5.3 \times 10^{-7}$ M and $K_{d2} = 1.1 \times 10^{-5}$ M. The dissociation reaction followed first order kinetics in two steps. The first step was very rapid, the second step proceeded with a half life time $t_{1/2}$ of 16 min and a dissociation rate constant of $k_{-1} = 7.2 \times 10^{-4}$ s^{-1}. The binding was sensitive to heat and SH-blocking reagents and displaceable by serotonin in excess, d-LSD, and to a lower extent by 5-methoxytryptamine and tryptamine.

The significance and localization of the binding sites at the membrane are discussed.

Introduction

Serotoninergic neurons were demonstrated in histochemical studies more than one decade ago (*Dahlström, Fuxe,* 1964), but till now little is known about the compartmentalisation within the nerve

ending and about the nature of the factors which regulate the local concentrations of serotonin (5-HT) or modify specific membrane responses to 5-HT within the synaptic junction. Recently a soluble protein with high binding affinity for 5-HT was found to be present in the cytoplasma of pinched-off nerve endings (*Tamir, Huang,* 1974). This protein possibly acts as carrier mediating the transport of 5-HT from the perikaryon to the presynaptic bouton. At the presynaptic membrane a transport mechanism is located which facilitates the retrieval of 5-HT released into the synaptic cleft. The kinetics of 5-HT uptake were studied in brain slices and isolated nerve endings (*Shaskan, Snyder,* 1970; *Ross, Renyi,* 1969; *Blackburn et al.,* 1967; *Horn,* 1973). In the present paper 5-HT binding proteins from nerve ending membranes were solubilized with an aqueous buffer system in order to study the binding kinetics of the particle free extract and to characterize the specificity and chemical nature of the binding.

Material and Methods

Chemicals. 5-Hydroxy[side chain-2-^{14}C]-tryptamine creatinine sulfate, [^{14}C]5-HT (specific activity 58 mCi/mmol), was purchased from Amersham Buchler, Braunschweig, Germany. Unlabelled 5-hydroxytryptamine creatinine sulfate, 5-HT, tryptamine hydrochloride, pronase (from Streptomyces griseus, EC 3.4.24.4, activity approx. 1.5 U/mg) and charcoal were from Merck, Darmstadt, Germany, and 5-methoxytryptamine, 5-MOT, and N-ethylmaleimide from Fluka AG., Buchs, Switzerland. d-Lysergic acid diethylamide tartrate, d-LSD, was generously provided by Sandoz AG., Nürnberg, Germany. Iodoacetamide and p-chloromercuriphenylsulfonic acid were obtained from Sigma Comp., St. Louis, U.S.A. Mulfogen BC-720 was a gift from GAF Corp., New York, and Vibrio cholerae neuraminidase [mucopolysaccharide-N-acetylneuraminylhydrolase EC 3.2.1.18, activity 500 u/ml according to *Mohr* and *Schramm* (1960)], was kindly provided by Behringwerke AG., Marburg, Germany. Dextran 500 was a product of Pharmacia, Uppsala, Sweden. All other chemicals were of analytical grade.

Animals

Male albino rats of the Wistar II strain (160—180 g) were used throughout. The animals were allowed to have free access to diet and water. The rats were killed by decapitation, and brains were quickly removed and placed into 0.32 M sucrose for further preparation.

Preparation of Synaptic Membranes

Nerve ending membranes were isolated from rat brain homogenates by differential and sucrose density gradient centrifugation (*Wesemann,* 1969).

All steps were performed at +4 °C. The purity of the preparations has been tested by electron microscopy. Contamination by mitochondria was also followed by enzyme analysis using succinate dehydrogenase, SDH (succinate: [acceptor] oxidoreductase, EC 1.3.99.1) as marker enzyme (*Kadenbach*, 1964).

Preparation of Soluble Membrane Extracts

5-HT binding proteins were extracted from synaptic membranes by stirring with 0.1 M sodium chloride in 1.0 mM Tris-HCl buffer, pH 7.4, containing 0.1 % Mulfogen BC-720 at +4 °C for 5 hours, followed by centrifugation at 100,000 x g_{max} for one hour in a Beckman preparative ultracentrifuge.

The resulting clear, yellowish supernatant was concentrated by ultrafiltration (Amicon UF cell, Diaflo membranes) at 50 psi and +4 °C to a protein concentration of 1.2—1.5 mg/ml and stored at 0 °C until use.

Assay of the [^{14}C]5-HT Protein Complex

The charcoal absorbent technique, introduced by *Heyns et al.* (1967) and *Korenman* (1968) for the study of steroid protein interaction has been evaluated and applied to 5-HT binding studies. 50 μl aliquots of the membrane extract were incubated at +4 °C with [^{14}C]5-HT at final concentrations ranging from 5×10^{-8} M to 9×10^{-6} M each in the presence or absence of unlabelled 5-HT in excess. After one hour 5-HT bound and unbound were separated by adsorption of the free 5-HT to dextran-coated charcoal (6 % [w/v]), added in 50 μl buffer.

The samples were agitated with a Cenco whirlmix for 10 s and then allowed to stand. If not otherwise indicated an incubation time of 5 min with charcoal was used throughout for routine assay. After centrifugation (2500 x g_{max}, 2 min) 50 μl of the clear supernatant were counted for activity in 15 ml of the scintillation fluid according to *Bray* (1960) by means of a Packard Tri-carb liquid scintillation spectrometer, mod. 3380/544. Drugs studied as competitors of 5-HT binding were added to the membrane extract simultaneously with [^{14}C]5-HT at zero time using concentrations as indicated in results. When sulfhydryl reagents were used at a final concentration of 1 mM, the incubation with [^{14}C]5-HT was preceded by a preincubation period of 1 hour at +4 °C.

In order to study thermal denaturation, aliquots of the membrane extract preparation were heated in a water bath at 30, 50, 55, 60, 65, 70, 80, 90, 100 °C for 10 min. After cooling to 0 °C precipitated material was removed by centrifugation and the resulting supernatants were used for 5-HT binding. Control samples were incubated simultaneously at +4 °C for 10 min and assayed together with the heat-treated preparations. In order to evaluate the time course of the displacement of bound [^{14}C]5-HT by unlabelled 5-HT and to determine the rate constant of dissociation the membrane extract was preincubated with 1.4×10^{-6} M [^{14}C]5-HT at +4 °C for one hour. Thereafter a 1000-fold excess of unlabelled 5-HT, 10 μl, or

buffer solution was added. This incubation lasted for various periods as mentioned under results. The incubation was stopped by addition of 50 μl of dextran-coated charcoal and agitation for 5 s. The charcoal was precipitated by centrifugation and the residual bound [^{14}C]5-HT of the supernatant was assayed for radioactivity. The time dependence of [^{14}C]5-HT binding to membrane extract was investigated as follows: 50 μl aliquots of the extract were incubated with 3.4×10^{-7} M [^{14}C]5-HT at +4 °C in the absence or presence of a 1000-fold excess of unlabelled 5-HT for varying times. After the indicated time periods (Fig. 3), 50 μl charcoal were added, followed by agitation for 5 s and centrifugation. The effects of enzyme treatment upon [^{14}C]5-HT binding activity were studied after a preincubation period of 30 min at 30 °C. The final concentrations of neuraminidase and pronase used per ml were 160 and 1.5 units, respectively. As tested in control assays without membrane extract, the enzymes alone did not bind 5-HT at these concentrations.

Enzyme Assay

The activity of monoamine oxidase (monoamine: oxygen oxidoreductase [deaminating], EC 1.4.3.4., MAO) was assayed according to *McCaman et al.* (1965) with the minor modification that the sample volumes were increased by factor 10. Control experiments with brain homogenates in detergent buffer and detergent free buffer were run in parallel in order to exclude a possible influence of the detergent on the enzyme activity.

Protein Determination

Protein was determined by the method of *Lowry et al.* (1951) using bovine serum albumine as a standard. In experiments with detergent buffer, a precipitate deposited during the formation of the coloured complex and had to be removed by centrifugation at 2500 x g for 10 min before recording the absorbance. Buffer containing detergent in the same concentration as used for solubilization was added to the albumine standards. The calibration curve of the detergent albumin system was linear.

Results

Validity of the Assay

Until now the charcoal method for rapid and gentle separation of free from bound ligands has not been used in studies with 5-HT. Thus, first the applicability of this method to 5-HT had to be evaluated. The adsorption capacity of the charcoal towards [^{14}C]5-HT was studied in the absence or presence of unlabelled ligand. The influence of the unlabelled 5-HT was tested up to a 5000-fold excess. Within this concentration range always about one

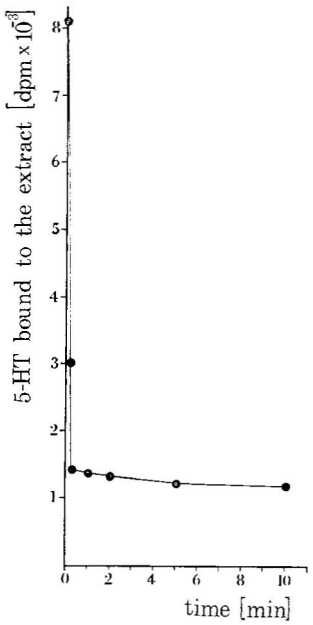

Fig. 1. Time dependence of 5-HT binding to charcoal. Membrane extracts (2.35 mg protein/ml, 50 µl) were incubated with 1×10^{-6} M [^{14}C]5-HT at +4 °C for 60 min. After addition of 50 µl of dextran-coated charcoal (6 % w/v) the samples were agitated for 10 s and incubated for the times indicated on the abscissa

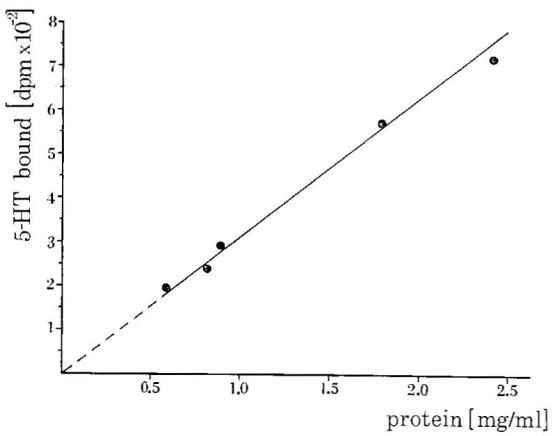

Fig. 2. 5-HT binding as function of protein concentration. Membrane extract was incubated with 3.4×10^{-7} M [^{14}C]5-HT at +4 °C for 60 min. The abscissa indicates the protein concentration of the protein solutions from which 50 µl aliquots were assayed

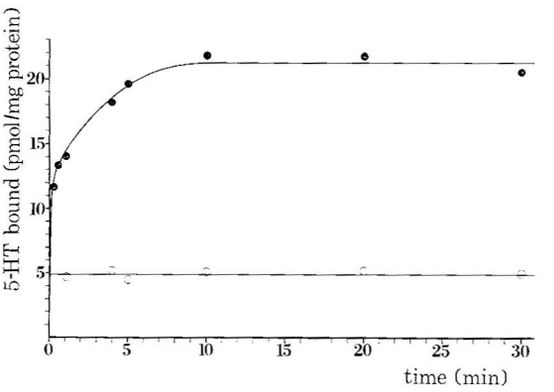

Fig. 3. Time course of [^{14}C]5-HT binding to membrane extract. 50 μl of a membrane extract (1.45 mg protein/ml) were incubated with 3.4×10^{-7} M [^{14}C]5-HT at +4 °C for varying times. Non-specific binding (○——○), *i.e.* binding in the presence of excess of unlabelled 5-HT, was not time dependent, in contrast to specific binding (●——●)

per cent of the total radioactivity applied to the incubate was found in the supernatant. This means that the charcoal is not saturated or overloaded with 5-HT under the experimental conditions employed. The dissociation of [^{14}C]5-HT bound to the membrane extract was measured as a function of the incubation time with charcoal. After adding the charcoal suspension to the samples followed by agitation for 10 s the incubation lasted various time periods. Figure 1 illustrates that within a few seconds equilibrium is obtained between free [^{14}C]5-HT and [^{14}C]5-HT bound to charcoal and to the membrane extract, respectively. Longer incubation times result in small or no changes of radioactivity in the supernatant. Thus a prolonged incubation with charcoal is unlikely to destroy the binding sites. Within the tested range (0.5—2.5 mg/ml) the amount of bound [^{14}C]5-HT correlated linearly with the protein concentration (Fig. 2). The possibility that adsorption of [^{14}C]5-HT into micelles of the detergent may increase the radioactivity found in the fraction of the membrane extract can be excluded since solutions of the detergent in buffer did not compete with charcoal for [^{14}C]5-HT binding. In addition in gel chromatographic studies radioactivity was eluted in the same fractions as the protein peak (unpublished results).

MAO Assay

The MAO activity was assayed in whole brain homogenates and in the membrane extract used for binding studies. In contrast to the

homogenate ($K_m = 4.5 \times 10^{-4}$ M, unpublished) there was no MAO activity found to be associated with the membrane extract.

Kinetics

Time Course of [^{14}C]5-HT Binding

When the membrane extract was incubated with [^{14}C]5-HT, the bound radioactivity representing the specific binding increased till a plateau was reached within 10 min (Fig. 3). In contrast to the specific binding a time independent linear relationship was obtained for the non-specific part of binding in the presence of excess unlabelled 5-HT.

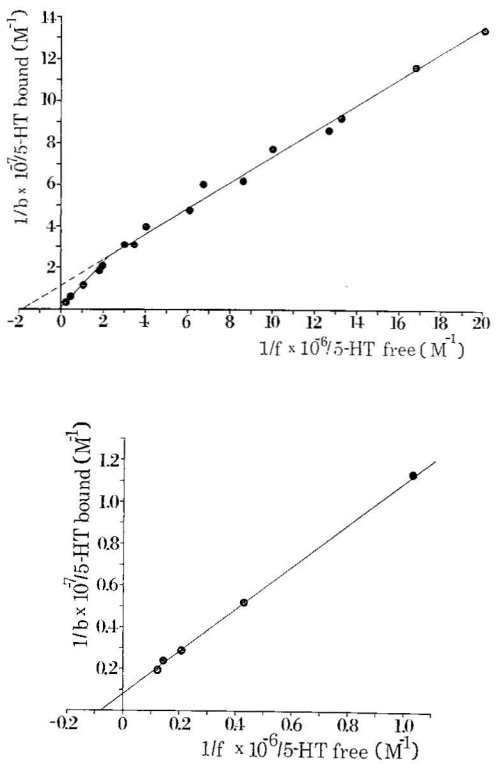

Fig. 4. *Lineweaver-Burk* plot of [^{14}C]5-HT binding to membrane extract as a function of 5-HT concentration. Membrane extract was incubated in quadruplicates with [^{14}C]5-HT concentrations ranging from 5×10^{-8} M to 9×10^{-6} M for one hour at +4 °C. The abscissa indicates reciprocals of [5-HT] free (1/f), the ordinate reciprocals of [5-HT] bound (1/b). Specific binding at [^{14}C]5-HT concentrations ranging from 5×10^{-8} M (Fig. 4 a) and from 9×10^{-7} M (Fig. 4 b) to 9×10^{-6} M

Saturation Kinetics

With increasing [^{14}C]5-HT concentrations saturable binding was observed when the amount of [^{14}C]5-HT bound in the presence of excess unlabelled 5-HT was substracted from the amount bound in the absence of unlabelled 5-HT. Similar results were obtained by a second method using the *Michaelis-Menten*-plot (5-HT bound, b, versus free, f). In this graph the linear, non-saturable part at high free ligand concentrations was extrapolated to zero concentrations and each of the values obtained was substracted from the total bound radioactivity. This specific binding was displaceable by 5-HT and reached a maximum whereas the non-specific binding increased linearly with increasing [^{14}C]5-HT concentrations. The double reciprocal plot 1/b versus 1/f according to *Lineweaver* and *Burk* (1934) gave a curvilinear relationship which could be resolved in two straight lines suggesting different binding affinities (Fig. 4 a). The higher affinity could be observed at ligand concentrations lower than 6×10^{-7} M. The apparent dissociation constants were calculated as $K_{d1} = 5.3\times10^{-7}$ M for the high affinity (Fig. 4 a) and $K_{d2} = 1.1\times10^{-5}$ M for the low affinity binding (Fig. 4 b) by extrapolating the linear parts of the plot to 1/b = 0. The finding that the membrane extract exhibits two different binding sites was also confirmed by plotting the data according *Scatchard* (1949). Again a curvilinear graph was obtained (Fig. 5), indicating two different classes of binding sites.

Time Course of [^{14}C]5-HT Displacement

Figure 6 illustrates the time dependence of the dissociation of the complex between 5-HT and membrane extract. The data were obtained by relaxation technique, *i.e.* measurements of [^{14}C]5-HT bound in the presence of competing unlabelled 5-HT as function of time. The dissociation reaction follows first order kinetics in two steps. The first step was too rapid to be acurately measured. The second step proceeds with a half life time $t_{1/2}$ of 16 min and a dissociation rate constant of $k_1 = 7.2\times10^{-4}$ s^{-1}.

Thermal Stability

The binding activity was found to be heat stable up to approximately 40 °C. Exposure to higher temperatures (50—60 °C) caused a rapid inactivation.

Chemical Characterization

Sulfhydryl Reagents

Since various receptors are inhibited by sulfhydryl blocking reagents we studied the influence of some of these substances on the binding of [^{14}C]5-HT (Table 1). The binding was markedly inhibited (about 55 %) especially by treatment with p-chloromercuriphenyl sulfonic acid. The decrease in [^{14}C]5-HT binding caused by N-ethylmaleimide was about 45 %. Iodoacetamide had no effect within

Table 1. *Effect of sulfhydryl-blocking reagents on [^{14}C]5-HT binding*

Compound added	[^{14}C]5-HT bound %
none (control)	100.0 ± 3.5
N-Ethylmaleimide	54.5 ± 3.7
p-Chloromercuriphenyl sulfonic acid	45.7 ± 3.6
Iodoacetamide	103.5 ± 3.6

Aliquots of membrane extract were preincubated for one hour at +4 °C with solutions of the indicated sulfhydryl reagents at a final concentration of 1×10^{-3} M. Thereafter [^{14}C]5-HT binding was assayed. The values indicate means of quadruplicate experiments ± S.D.

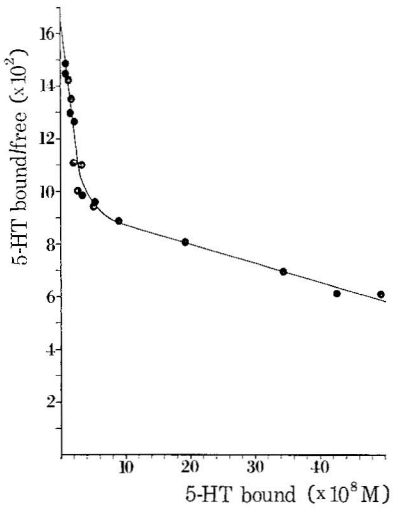

Fig. 5. *Scatchard* plot of [^{14}C]5-HT binding to membrane extract as a function of 5-HT concentration. The preparation of the extract and the [^{14}C]5-HT concentrations are identical to those described in the legend of Fig. 4

the time of incubation. These results indicate that SH-groups are essential constituents of 5-HT binding to membrane extract.

Enzyme Treatment

Specific [^{14}C]5-HT binding was reduced to about 89 % by neuraminidase, while pronase caused a marked decrease of 71 %.

Effect of 5-HT Structural Analogues on [^{14}C]5-HT Binding

The specificity of the 5-HT binding to membrane extract was studied with substances of known pharmacological antagonism in electrophysiological experiments and competitors in uptake kinetics of synaptosomes (Table 2). d-LSD was the most potent competing substance, lowering the amount of bound 5-HT by 55 % of the control. The inhibition by 5-methoxytryptamine or tryptamine was in the range of 20 %.

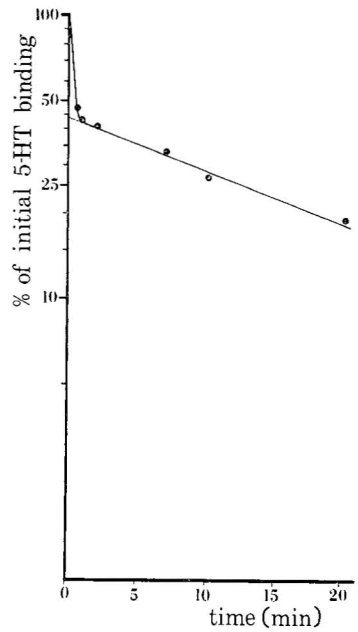

Fig. 6. Time course of displacement of bound [^{14}C]5-HT by unlabelled 5-HT in excess. Aliquots of membrane exctract (50 μl) were incubated with [^{14}C]5-HT 1.4×10^{-6} M at +4 °C for one hour. At zero time unlabelled 5-HT was added in excess. The samples were incubated for varying times as indicated. The incubation was stopped by the addition of charcoal in order to separate free from the residual bound 5-HT. The values give the bound [^{14}C]5-HT in per cent of control (without unlabelled 5-HT) on a logarithmic scale

Table 2. *Effect of 5-HT analogues on [^{14}C]5-HT binding*

Ligand in excess	[^{14}C]5-HT bound %
none (control)	100.0 ± 5.4
d-LSD	44.6 ± 6.7
5-MOT*	80.4 ± 7.7
Tryptamine	81.0 ± 3.3

Aliquots of membrane extract were incubated simultaneously with 3.4×10^{-7} M [^{14}C]5-HT and the indicated unlabelled compounds in 1000-fold excess. The values indicate the residual 5-HT binding in presence of analogue substances expressed as per cent ± S.D. of specific binding of control samples in absence of competing compound.

* MOT: 5-methoxytryptamine

Discussion

Brain slices or synaptosomes were the object of numerous studies for accumulation of 5-HT. As shown by the unsuccessful attempts of *Robinson et al.* (1965) and *Schanberg* (1963) saturable specific uptake of 5-HT is limited to low 5-HT concentrations within the incubation medium. With high 5-HT concentrations only a non-specific diffusion could be observed, increasing linearly with total 5-HT. In this case, the specific process is totally masked by non-specific hydrophobic, ionic binding or cation exchange effects (*Robinson et al.*, 1965). The K_d-values of the specific uptake mechanism given in the literature are mostly below 1 μM. If two affinities were found, the significance of the second uptake site has been interpreted differently. *Shaskan* and *Snyder* (1970) suggested that the lower affinity ($K_d = 8 \times 10^{-6}$ M) was related to a catecholamine uptake system, whereas *Marchbanks* (1966) described a medium affinity ($K_d = 2 \times 10^{-5}$ M) inhibited by iproniazide and harmine. *Farrow* and *Van Vunakis* (1973) reported two binding sites for d-LSD at isolated synaptosomal membranes ($K_{d1} = 9 \times 10^{-9}$ M, $K_{d2} = 1.2 \times 10^{-6}$ M). In our study with solubilized proteins from synaptic membranes we found two classes of binding sites for 5-HT with the apparent dissociation constants of $K_{d1} = 5.3 \times 10^{-7}$ M and $K_{d2} = 1.1 \times 10^{-5}$ M.

Only few attempts have been described to solubilize the membrane linked 5-HT binding sites for further characterization. *Marchbanks* (1966) reported that the high affinity sites ($K_d = 5 \times 10^{-7}$ M) of

synaptosomes could only be extracted with butanol but not with detergent containing buffers. These high affinity sites were destroyed by neuraminidase. Although the 5-HT binding to pure gangliosides was weaker than that observed with the butanol extract, he concluded that gangliosides are at least partly involved in the binding to nerve membranes. Gangliosides as 5-HT receptors were also discussed by *Van Heyningen* (1974). *Fiszer* and *De Robertis* (1969) also studied the 5-HT binding to a butanol extract using 5-HT concentrations higher than 1 μM. They suggested that the 5-HT binding substance is a special type of a proteolipid not present in myelin. Our findings obtained with low concentrations of 5-HT after preincubation with neuraminidase cannot refute that 5-HT binding may be related to gangliosides. Further work has to clarify the significance of neuraminidase treatment. In a combined electron microscopic and 5-HT binding study of synaptic structures *Wesemann et al.* (1971) provided evidence that possibly sialoglycolipids or sialoglycoproteins may act as 5-HT receptor molecules. The data reported in the present paper are in favour of the latter view.

The binding activity was found to be heat stable up to approximately 40 °C, as reported similarly by *Farrow* and *Van Vunakis* (1973) for d-LSD binding sites at preserved synaptic membranes. Exposure to higher temperatures caused a rapid inactivation. The binding sites were further characterized by means of SH-blocking reagents and pronase incubation. The 5-HT binding was decreased by both treatments, which agrees well with the assumed protein nature of the 5-HT acceptor molecule.

The receptor model calculated on the preferred 5-HT conformation requires the interaction with the three heteroatoms of the serotonin molecule (*Kier*, 1968). The binding of d-LSD to the receptor has been explained by interaction with only two of them, the protonated N-methyl group and the pyrrol nitrogen. In the present study we found that d-LSD was the most potent 5-HT competing ligand. This specificity correlates well with electrophysiologic findings, inhibition studies of 5-HT binding and vice versa, inhibition of LSD-binding by 5-HT (*Farrow, Van Vunakis*, 1973; *Aghajanian et al.*, 1972; *Bennet, Aghajanian*, 1974; *Marchbanks*, 1966). The importance of the oxygen at position 5 is evident, since 5-HT and tryptamine effects are distinguishable in electrophysiologic and in vitro studies. Depending on the experimental conditions employed 5-methoxytryptamine and tryptamine showed either different or similar effects on the affinity (*Horn*, 1973; *Ross, Renyi*, 1969; *Farrow, Van Vunakis*, 1973). In our system no difference was found between these two substances which were much weaker inhibitors of 5-HT

binding than d-LSD. At present it is not possible to decide whether the solubilized 5-HT binding sites are located pre- or postsynaptically at the membrane. d-LSD binds to both membranes, but as it has been assumed, predominantly to the postsynaptic membrane (*Bennet, Aghajanian,* 1974). Different data are found in the literature about the inhibitory action of d-LSD on 5-HT uptake or binding sites. *Ross* and *Renyi* (1967) did not find any significant inhibition of 5-HT uptake into brain slices by LSD, while the 5-HT binding to butanol extracts of synaptosomes was inhibited (*Marchbanks,* 1966). The assumption of acceptor sites, differing in sensitivity, may possibly explain these conflicting results: while the receptors at the membrane (pre- or postsynaptically) are occupied by d-LSD, the capacity of the hypothetical carrier in the presynaptic membrane is not affected.

Summarizing our experimental findings together with data of the literature as discussed above: first, the results of the physical and chemical treatment give evidence that the chemical nature of the 5-HT binding molecule is that of a protein; second, the different inhibitory effects of 5-HT analogues suggest that the binding analysed in the present paper may represent interaction with a receptor which is located at the membranes of serotoninergic nerve endings and is different from the carrier system.

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