Abstract—To investigate the nature of the tryptamine binding components that originated from myelin butanol extracts (i.e., myelin proteolipids), the lipid mixtures obtained from these extracts were further fractionated by silicic acid column chromatography, and binding assays of $^{14}$C-tryptamine to those fractions were carried out by Sephadex LH$_{20}$ column chromatography. Among several lipid fractions, only the F-C fraction retained the tryptamine binding properties of the original myelin butanol extracts, i.e., binding capacity, chromatographic profile and interaction with indoleamine analogues and other neurotransmitters. Since the quantitative TLC analysis indicated that this fraction contained a considerable amount of phosphatidylserine (PS) and phosphatidylinositol (PI), recombination experiments with these two acidic lipids were planned. The recombination system with PI did not show a tryptamine binding capacity, while the PS fraction possessed a tryptamine binding capacity similar to that of the myelin butanol extracts. However, displacement studies revealed that the recombinant fraction with PS alone did not display the complete regeneration of the specificity which had been observed in the myelin extracts. All these observations infer that the tryptamine binding components from myelin proteolipids are lipid in nature, and its binding entity is mainly PS. However, some specifically organized constitution with PS and other lipid molecule(s) may be necessary to regenerate the original tryptamine binding properties.

The proteolipids of Folch-Lees (1), which appear to occur in membraneous structures, have the unusual property of being soluble in chloroform-methanol or butanol-water mixtures, but are insoluble in water. Recently, several investigations (2-5) have implicated the proteolipids-like protein in the binding of neurotransmitters and drugs to lipid extracts of nervous tissue. On the other hand, it has also been indicated that what has been reported to be a possible proteolipid opiate receptor involves the acidic lipid sulphatides (6). Furthermore, Johnson et al. (7) have pointed out that 5-hydroxytryptamine (5-HT) binds saturaingly and with high affinity to acidic lipids.

In previous studies (8), we found that the myelin butanol extracts (i.e., myelin proteolipids) had a specific binding capacity for tryptamine as well as 5-HT (9), and moreover, its binding components may be lipid in nature. The objective of the present work was to investigate the nature of the tryptamine binding components from myelin proteolipids in detail.

Materials and Methods

Chemicals: Sephadex LH$_{20}$ was obtained from Pharmacia. The silicic acid was Mallinckrodt's analytical reagent. DEAE cellulose was obtained from Brown. Indoleamine analogues and neurotransmitters were from the Sigma Chemical Co. Tryptamine-[2-$^{14}$C] bisuccinate (51.5 mCi/mmol), 5-HT-[1,2-
Preparation of lipid fractions: Myelin butanol extracts were prepared from rat brain stems (male Wistar, 150–200 g) as reported previously (8). Briefly, myelin fragments were isolated from the homogenate by the method of Norton and Poduslo (10) and extracted with water-saturated butanol. Just before use, these extracts were treated with water (14%, v/v) to dissolve the precipitated materials (total extracts=TE). Lipid mixtures were obtained from TE by the ice-cold ether treatment method of Mokrasch (11). Several lipid fractions were isolated from these mixtures by silicic acid column chromatography (12). Namely, an aliquot of sample (approx. 15 μmoles as lipid phosphorus) was passed through a silicic acid column (1.3×15 cm), and each fraction was fractionated with the following scheme:

| Fraction | Solvent composition (chloroform-methanol, v/v) | Total volume (ml) |
|----------|-----------------------------------------------|-------------------|
| F-A      | 98:2                                          | 30                |
| F-B      | 90:10                                         | 60                |
| F-C      | 80:20                                         | 40                |
| F-D      | 10:100                                        | 40                |

The isolated lipid fractions were evaporated under N₂, and the resultant residues were dissolved in butanol-water mixtures (14%, v/v) for use in binding experiments.

Binding of 14C-tryptamine: Aliquots of each sample (0.3 ml) were incubated at room temperature for 20 min with 5×10⁻⁷ M 14C-tryptamine. After incubation, the mixtures were loaded onto a Sephadex LH₂₀ column (0.6×19 cm), and stepwise elution was carried out with the following solvents: 10 ml of chloroform and chloroform-methanol (CM) 10:1 and then 16 ml of CM 9:1. After chromatography, an aliquot of each of the collected fractions (0.4 ml) was added to counting vials containing 10 ml of toluene/ Triton X-100 emulsion phosphor (13), and radioactivity was measured in a liquid scintillation counter. In this elution system, a minute amount of the free ligand (approx. 5%) appeared in the binding peak. Thus, the amount of bound tryptamine was calculated by the subtraction of free portion from the total binding.

Lipid analysis: Cholesterol was directly analyzed according to the method of Searcy and Bergquist (14). Individual phospholipids were determined by the quantitative TLC method of Norton and Autilio (15) using a two dimensional solvent system: chloroform-methanol-ammonium hydroxide (14:6:1) followed by chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1). Total lipid phosphorus was assayed by the method of Chen et al. (16). Minor galactolipids, cerebrosides and sulphatides were also estimated by a quantitative TLC method. Briefly, an aliquot of sample (approx. 2 μmoles as lipid phosphorus) was spotted on a TLC plate. The plate was developed in chloroform-methanol-water (70:30:4), and lipids were visualized with iodine vapor. The spots corresponding to each galactolipid were scraped off the plate, extracted with CM 1:2, and the galactose concentration was determined by the method of Svennerholm (17). To calculate the molar concentration of each lipid, the following average molecular weights were used: minor galactolipids, 846; cerebrosides, 828; sulphatides (CS), 933; ethanolamine phosphatides, 732; choline phosphatides, 804; sphingomyelin, 800; phosphatidylserine (PS), 820 and phosphatidylinositol (PI), 890.

Preparation of phosphatidylserine and phosphatidylinositol: PS and PI were separated from rat brain stems by silicic acid and DEAE cellulose (acetate form) column
chromatographies as described previously (18). Both lipids were better than 96% pure.

Results

In a preliminary experiment, we found that the lipid mixtures prepared from myelin butanol extracts retained a binding capacity for 14C-tryptamine. Thus, as a first step toward the examination of the nature of tryptamine binding components present in these extracts, lipid mixtures were further fractionated into 4 fractions by silicic acid column chromatography, and the 14C-tryptamine binding capacities of these fractions were checked. As shown in Table 1, only the F-C fraction had a tryptamine binding capacity similar to that of the original myelin extracts. Furthermore, 14C-tryptamine elution profiles of myelin butanol extracts, lipid mixtures and F-C fraction were examined by Sephadex LH20 column chromatography (see Fig. 1). These three preparations displayed similarities with respect to their chromatographic properties.

In previous studies (8), it was demonstrated that only tryptamine and 5-methoxytryptamine inhibited the 14C-tryptamine binding to myelin butanol extracts, but indoleamine analogues and other neurotransmitters had no effect. To clarify whether or not the lipid mixtures and the F-C fraction represent the above mentioned specificity, displacement experiments were performed. The results (Table 2) indicated that both preparations completely regenerated the same specificity as that of the original butanol extracts. All these observations strongly suggest that the F-C fraction certainly reflects the nature of the tryptamine binding components from myelin proteolipids.

Before focusing on the investigation of the nature of tryptamine binding components upon some special lipid molecules, we analyzed the lipid composition of several fractions which retain a specific binding capacity for 14C-tryptamine. Results are presented in Table 3. Since the acidic lipids are tightly associated with the basic proteins

![Fig. 1. Sephadex LH20 chromatograms of myelin butanol extracts, lipid mixtures and F-C fraction. Samples were incubated with 5×10^{-7} M 14C-tryptamine. The discontinuous elution system described in the text was used.](image-url)

Table 1. 14C-Tryptamine binding to several fractions

| Fraction       | Amount of tryptamine bound (%) |
|---------------|--------------------------------|
| Butanol extracts       | 72.2±6.4*            |
| Lipid mixtures            | 71.8±5.3*            |
| F-A                    | None                |
| F-B                    | None                |
| F-C                    | 72.3±5.0            |
| F-D                    | None                |

Several fractions were incubated with 5×10^{-7} M 14C-tryptamine. After incubation, the mixtures were passed through a Sephadex LH20 column, and the bound radioactivity was measured as described in the text. Results are expressed as the percent of total input radioactivity (mean±S.E.M. of 3 experiments). *Data from Ref. (8).
which are included in the myelin butanol extracts (19), the contents of these acidic lipids in lipid mixtures (i.e., CS, PS and PI) were generally lower than those of the original butanol extracts. If it is possible to assume that the lipid groups of the F-C fraction which are present at concentrations as little as 10% of those in the myelin extracts are not implicated in the tryptamine binding, only the ethanolamine phosphatides, PS and PI, would be candidates for the entity involved as the tryptamine binding component. On the other hand, as pointed out by Johnson et al. (7), an electrostatic interaction between tryptamine and lipid molecules is the most likely binding mode. Based on these assumptions, we arbitrary selected the acidic lipids, i.e., PS and PI, as the nature of the tryptamine binding components. The recombinant fractions of PS and PI alone (20 and 3 μg, respectively) were incubated with 5×10⁻⁷ M ¹⁴C-tryptamine and binding capacities of both were examined by Sephadex LH₂₀ column chromatography. Only the recombinant fraction with PS possessed a tryptamine binding capacity (72.0±5.0% of input total radioactivity, mean±S.E.M. of 3 experiments) similar to that of the myelin butanol extracts, while PI alone had no binding capacity. Furthermore, as shown in Figs. 1 and 2, myelin butanol extracts and the recombinant of PS also showed similarities with respect to their mutual chromatographic properties. However, displacement experiments revealed that the PS alone and PS+PI could not completely regenerate the specificity to indoleamine analogues and other neurotransmitters which

| Compound                          | Lipid mixtures | F-C fraction  | Butanol extracts* |
|-----------------------------------|----------------|---------------|-------------------|
| Tryptamine                        | 84.5±3.8       | 85.9±4.0      | 59.4±12.3         |
| 5-Hydroxytryptophan               | None           | None          | None              |
| 5-Hydroxy-3-indole acetic acid    | None           | None          | None              |
| 5-Methoxytryptamine               | 100            | 100           | 78.9±4.0          |
| 5-Hydroxytryptamine               | None           | None          | None              |
| Acetylcholine                     | None           | None          | None              |
| Dopamine                          | None           | None          | None              |
| Noradrenaline                     | None           | None          | None              |

Samples were preincubated with various compounds (all: 5×10⁻⁴ M) for 30 min, and then 5×10⁻⁷ M ¹⁴C-tryptamine was added. After incubation, the bound radioactivity was measured by Sephadex LH₂₀ column chromatography as described in the text (mean±S.E.M. of 4 experiments). *Data from Ref. (8).

| Lipid                          | Lipid mixtures | F-C fraction | Butanol extracts |
|-------------------------------|----------------|--------------|------------------|
| Cholesterol                   | 469.9±14.0     | Not detected | 490.0±10.0       |
| Minor galactolipids           | 23.3±2.0       | 1.4±0.5      | 21.0±0.8         |
| Cerabrosides                  | 226.4±4.0      | 6.5±0.4      | 306.7±7.6        |
| Sulphatides                   | 7.0±0.6        | 4.0±0.2      | 79.0±1.7         |
| Ethanolamine phosphatides     | 310.3±7.0      | 301.8±6.2    | 303.1±3.3        |
| Choline phosphatides          | 189.9±5.7      | Not detected | 192.3±4.7        |
| Sphingomyelin                 | 16.4±0.9       | 1.3±0.2      | 38.5±3.8         |
| Phosphatidylserine            | 31.7±0.5       | 19.2±1.2     | 76.1±4.2         |
| Phosphatidylinositol          | 4.2±0.6        | 2.8±0.1      | 8.6±0.3          |

Values are expressed as μg/incubation mixture (mean±S.E.M. of 4 determinations).

Table 2. Inhibition of ¹⁴C-tryptamine binding by various compounds

Table 3. Lipid compositions
had been observed in the myelin butanol extracts (data is not presented).

In addition, we have already reported that tryptamine inhibited 5-HT binding to myelin extracts (9), but the vice versa was not observed (8). To investigate the interaction between the tryptamine and 5-HT binding components, double labelling experiments were performed by Sephadex LH$_{20}$ column chromatography. The elution peak of $^{14}$C-tryptamine appeared in the boundary between CM 6:1 and 4:1 as reported previously (8), whereas $^3$H-5-HT was eluted in the different fraction of CM 4:1 (see Fig. 3). These results support that the binding components of both amines may differ.

Discussion

The cold-ether treatment method of Mokrasch (11) selectively disrupts the association between protein and lipid moieties of proteolipids. When the myelin butanol extracts (i.e., myelin proteolipids) were treated with 4 vol. of ice-cold ether, 6.1 and 82.1% of protein and lipid phosphorus were recovered in the supernatant fraction (i.e., lipid mixtures), respectively (9). In a preliminary experiment, we found that lipid mixtures had a $^{14}$C-tryptamine binding capacity, and moreover, the heat-treated myelin butanol extracts (80°C, 30 min) also retained the inherent tryptamine binding capacity. Thus, we focused the examination
of the nature of tryptamine binding components from myelin proteolipids on the lipid portion.

Lipid mixtures were fractionated into 4 fractions by the silicic acid column chromatography of Norton and Autilio (12). The quantitative TLC analysis indicated that each fraction contained mainly the following lipids: F-A fraction, cholesterol and minor galactolipids; F-B fraction, cerebrosides; F-C fraction, ethanolamine phosphatides, PS and PI; and F-D fraction, choline phosphatides and sphingomyelin. Among these four lipid fractions, only the F-C fraction possessed a tryptamine binding capacity similar to that of the original butanol extracts, and moreover, the elution profile of 14C-tryptamine binding to this fraction well corresponded to that of the myelin extracts. Furthermore, displacement studies with indoleamine analogues and other neurotransmitters revealed that this fraction also reflected the original specificity. All these observations strongly suggested that the F-C fraction retained the tryptamine binding components present in the myelin butanol extracts.

From the quantitative lipid analysis of three preparations, i.e., myelin butanol extracts, lipid mixtures and F-C fraction, it was found that the contents of ethanolamine phosphatides, PS and PI in the F-C fraction were not so low, but the contents of cholesterol, minor galactolipids, cerebrosides, CS, choline phosphatides and sphingomyelin were remarkably reduced in comparison with those of the two others. Although the primary role of lipids is to provide a molecular “cement” for the construction of biomembranes and other cellular organelles, some dynamic function of lipids could also be possibly considered. For example, Loh and Law (20) have pointed out that one of the dynamic functions of lipids in receptor mechanisms is to act directly as binding sites themselves, and the most likely candidates for this role are the acidic lipids since a large number of neurotransmitters and drug molecules are cationic under the physiological environment. In fact, several investigators have reported that acidic lipids are implicated in the proteolipids nature of the neurotransmitters and drug binding components (i.e., receptors) (6, 7, 20). From these observations, we designed recombination experiments with two acidic lipids, PS and PI. The recombination system with PI did not show any binding capacity for 14C-tryptamine, while the recombinant of PS possessed a tryptamine binding capacity similar to that of the original butanol extracts. However, displacement studies revealed that PS alone and PS+PI did not completely regenerate the specificity which had been observed in the myelin butanol extracts.

In addition, double labelling experiments with 14C-tryptamine and 3H-5-HT clearly demonstrated the difference between the binding components of both amines (see Fig. 3). From studies on the identification of the 5-HT binding components that originated from myelin proteolipids, it has been shown that the recombinant fraction of CS alone shows a 5-HT binding capacity similar to that of the original ones, but the presence of PS and PI besides CS is essential for the regeneration of the inherent specificity (18, 21).

Finally, the present results suggest that the tryptamine binding components of myelin proteolipids are lipid in nature, and its binding entity is mainly PS. However, some specifically organized construction with PS and other lipid molecule(s) may be necessary to display the specificity, as in the case of 5-HT. Since the neurophysiological function of myelin is not yet obvious, it is very difficult to imply the physiological relevance of these binding components. Nevertheless, it was intriguing to find the binding of tryptamine to specific lipids.
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