SEPARATION OF CHOLINERGIC PROTEOLIPIDS FROM QUENCHED RAT CEREBRAL CORTEX AND RESOLUTION OF PROPERTIES AT LOW TEMPERATURE

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Abstract—Proteolipids from rat cerebral cortex quenched at -196°C were extracted and fractionated at subzero temperature (-60°C). On Sephadex LH-20 column chromatography, acetylcholine and cholinergic blocking agents such as dimethyl-*d*-tubocurarine and decamethonium were observed to bind to different fractions of proteolipids showing that the receptor fractions for acetylcholine and that for cholinergic blockers need not be the same. The acetylcholinesterase (EC 3.1.1.7) activity was demonstrated to be absent in both types of receptor fraction. The specificities of binding by cholinergic substances to proteolipid fractions prepared at -60°C persisted at room temperature with some loss in specificity for acetylcholine. Since these specificities were not observed in previously reported experiments at room temperature, it appears that the structures of proteolipids extracted at the two temperatures differ. The appearance of specificities in the proteolipids prepared at low temperature suggest that their structures are in better approximation to those *in vivo* that presumably are highly specific. The importance of the protein moiety of proteolipids as a discriminator for the neurotransmitter is discussed.

Special hydrophobic proteolipids extractable with chloroform-methanol (2:1 v/v) have been reported to be found in large amounts in brain white and gray matter by Folch-Pi and Lees (1). De Robertis and his associates have demonstrated that proteolipids from mammalian brain showed high affinities for binding drugs affecting synaptic transmission, such as dimethyl-*d*-tubocurarine (2), atropine (3), serotonin (4) and several adrenergic blocking agents (5, 6). Such proteolipids were also isolated and purified from the electric organ of *Torpedo* and *Electrophorus* (7) and were shown to bind acetylcholine and cholinergic drugs, being designated as "receptor proteolipids". But this solvent for the extraction of proteolipids is well known for its modification of protein structure, not infrequently to the extent of irreversible denaturation.

To obtain preparations of proteolipids in better approximation to *in vivo* structure, extraction of the brain tissue, previously quenched at -196°C was carried out at -60°C . The binding abilities of such proteolipids for acetylcholine and cholinergic blocking agents were examined at low temperature and the characteristics of the proteolipids as the possible cholinergic receptor fractions are discussed.

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At the same time, the authors would like to introduce the technical details of low temperature which could be applied to studies in pharmacology.

MATERIALS AND METHODS

Preparation of proteolipids from rat cerebral cortex at low temperature

The cerebral cortex of Sprague-Dawley rats was quenched at -196°C in liquid nitrogen and pulverized at this temperature. The brain powder was stored at -78°C (dry ice temperature). The following procedures were all carried out below -60°C except column chromatography, at -60°C . Extraction of tissue to acquire a total lipid extract was done

FIG. 1. Diagram of filter for use at subzero temperature.

Container and well were precooled in dewar flask with dry ice (in methanol) to desired temperature (-60°C). Samples were applied in the well and then filtered slowly under low vacuum. The filtrate was recovered in the container.

FIG. 2. Diagram of an apparatus for molecular distillation at subzero temperature.

Samples for molecular distillation were put in the container precooled with dry ice as in Fig. 1. After connecting to a vacuum line which was driven by an oil pump and mercury diffusion pump, the O-ring valve was opened slowly. The well was then filled with liquid nitrogen, thus getting a high vacuum. Using this apparatus, 1 l of chloroform-methanol (2:1 v/v) was completely dried up in a day at -60°C .

with chloroform-methanol (2:1 v/v, 100 ml for 100 mg of brain powder). The extraction was carried out for 20 hours with mixing. The extract was freed from residue by filtration (Fig. 1). The organic solvent was completely removed under high vacuum by molecular distillation (Fig. 2). The dried material was dissolved with chloroform-methanol (2:1 v/v) and was used as total lipid extract for chromatography and for further isolation of proteolipids. Proteolipids were precipitated from the total lipid extract by adding twice its volume of diethyl ether (9). The precipitate was gathered by centrifugation and the organic solvents was removed under high vacuum. The proteolipids were finally dissolved with chloroform-methanol (2:1 v/v) and allowed to react with acetylcholine and cholinergic blocking agents.

Preparation of Sephadex LH-20 column

Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala) previously washed at room temperature according to the instruction of the manufacture except that methanol was substituted for acetone, was rinsed in pure chloroform (500 ml for 50 g of Sephadex LH-20) and allowed to stand for 5 hours at room temperature for complete swelling. It was then cooled down to -60°C with gentle stirring under low vacuum thus minimizing the dissolution of air into the liquid at this temperature. The cooled Sephadex LH-20 gel was packed into the column which was put in the refrigerator cabinet (Model ULT 657, Revco) adjusted at -60°C . To acquire the homogenous packing, the gel was first transferred to the flask attached to the top of the column and packed slowly with the aid of low vacuum.

On the top of the gel, 2 cm of ignited sea sand was put to avoid the floating of gel in pure chloroform. The column was washed with 150 ml of chloroform and allowed to stand overnight to get a constant flow rate. The column was freshly prepared for each experiment and the one used in this experiment had the dimensions, 1.8 cm $\times$ 45 cm and the gel was packed to a height of 40 cm. The outlet of the column made of glass tubing (2 mm caliber) ascended to a height 10 cm below the top of the gel. For the eluants, the fraction collector (ISCO Model 272) was also put in the refrigerator cabinet. For satisfactory running of the fraction collector, all grease at the gears was removed and a small electric heater was placed below the operating unit thermally insulated from cabinet air space.

Column chromatography at low temperature (-60°C)

Column chromatography at low temperature was used to detect the bindings of acetylcholine and cholinergic blocking agents to proteolipids preparation. Proteolipids (containing about 5 mg of protein) dissolved in 5 ml of chloroform-methanol (2:1 v/v) were incubated with ^{14}C -acetylcholine (5×10^{-8} moles), dimethyl- ^{14}C -*d*-tubocurarine (2×10^{-8} moles), ^{14}C -decamethonium (2×10^{-8} moles) or ^3H -atropine (0.5×10^{-8} moles) each of which was dissolved in 0.5 ml methanol. Incubation was carried out at -60°C for 1 hour. After incubation, the mixture was applied to the Sephadex LH-20 column and eluted with chloroform (120 ml) and chloroform-methanol (4:1 v/v, 120 ml) in succession. The eluant in 3.5 ml samples was then brought up to room temperature. Assay of the protein was done by the absorption at 280 m μ by a Cary 14 spectrophotometer. The total phosphorus was estimated according to the method of Martin and Doty (10), after digestion of the dried samples with perchloric acid. Radioactivity in each fraction was counted by liquid scintillation. The quenching of radioactivity by the presence of chloroform or methanol was corrected so as to obtain actual dpm. Acetylcholinesterase activity was estimated using acetylthiocholine as substrate and DNTB (Dithiobisnitrobenzoic acid) as indicator (11).

Biphasic experiments at room temperature

Biphasic distribution experiments were also done to detect the binding of radioactive acetylcholine and cholinergic blocking agents to proteolipids from rat brain.

Proteolipid preparations which were prepared at low temperature as described above were brought up to ice temperature and dissolved in chloroform-methanol (2:1 v/v). Three milliliters of this solution containing 30 μg of proteolipids as protein per milliliter were placed in screw topped testing tubes which were precooled to ice temperature. To this, one milliliter each of aqueous solution of ^{14}C -acetylcholine (2×10^{-5} M), dimethyl- ^{14}C -*d*-tubocurarine (2×10^{-5} M) or ^{14}C -decamethonium (2×10^{-5} M) was added and mixed vigorously for 2 min at room temperature using a Vortex JR mixer. The tubes were then centrifuged to obtain the clear separation of two phases, upper aqueous phase (2 ml) and lower organic phase (2 ml). Some parts of each phase were transferred to scintillation vials and counted for radioactivity.

Radioactive materials were from New England Nuclear (Boston, Massachusetts): acetyl-1- ^{14}C -choline iodide (4.53 mc/mM), decamethonium-1, 10- ^{14}C -dichloride (6.54 mc/

mM), dimethyl- ^{14}C -*d*-tubocurarine (6.16 mc/mM) and ^3H -atropine sulfate (890 mc/mM). The solutes, chloroform and methanol, were of spectrophotometric grade (Baker Chemical Co. New Jersey). Phosphatidyl serine of high purity had been freshly prepared by Applied Science Laboratories (State College, Pennsylvania) and similarly monophosphatidyl inositol by General Biochemicals (Chagrin Falls, Ohio).

RESULTS

Binding of ^{14}C -acetylcholine to total lipid extract and proteolipids isolated and incubated at -60°C

Fig. 3 represents the elution profile of total lipid extract (TLE) incubated with ^{14}C -acetylcholine at -60°C and applied to the Sephadex LH-20 column. Clearly, a coincidence occurred in the peak of acetylcholine with the second peak of proteolipids eluted with chloroform and also with the main peak eluted with chloroform-methanol (4:1 v/v), the more polar solvent.

FIG. 3. Elution of total lipid extract incubated with ^{14}C -acetylcholine at -60°C .

Total lipid extract containing 5 mg of protein in 5 ml of chloroform-methanol (2:1 v/v) was subjected to incubation with 2.5×10^{-8} moles of ^{14}C -acetylcholine and applied to a Sephadex LH-20 column (1.8–40 cm). Elution was carried out with pure chloroform followed by chloroform-methanol (4:1 v/v) as indicated by the arrow. Fractions were 3.5 ml per tube.

Radioactive acetylcholine (2.5×10^{-8} moles in 0.5 ml of methanol) applied at -60°C with 5 ml of chloroform to a Sephadex LH-20 column was totally bound to Sephadex and remained uneluted by chloroform. If to such a bound column, after being washed with 120 ml of chloroform, a bolus of proteolipids dissolved in 5 ml of chloroform-methanol (2:1 v/v) was similarly applied (delayed application), subsequent passage of chloroform gave a profile showing coincidence of ^{14}C -acetylcholine with the second peak of proteolipid (Fig. 4). When 5 ml of chloroform-methanol (2:1 v/v) was substituted for proteolipids solution, the ^{14}C -acetylcholine still remained tightly bound to the column in the course of elution by chloroform. Virtually the same elution pattern of column chromatography was produced by a solution of proteolipids incubated with ^{14}C -acetylcholine for 1 hour at -60°C .

FIG. 4. Delayed application of proteolipids to the Sephadex LH-20 column preloaded with 2.5×10^{-5} moles of ^{14}C -acetylcholine at -60°C .

The Sephadex LH-20 columns were preloaded with 2.5×10^{-5} moles of ^{14}C -acetylcholine and washed with 120 ml of pure chloroform. To such a column, proteolipids dissolved with 5 ml of chloroform-methanol (2:1 v/v) were applied. In control experiments, 5 ml of chloroform-methanol (2:1 v/v) were substituted for proteolipids solution. Subsequent procedures were the same as under Fig. 3.

The binding of ^{14}C -acetylcholine to Sephadex LH-20 column was dependent on the polarity of the elution solvent. Even in the absence of proteolipids, small amounts of ^{14}C -acetylcholine were loosened from the column by the more polar solvent, chloroform-methanol (4:1 v/v). But in the presence of proteolipids, the elution peak of ^{14}C -acetylcholine was shifted forward showing higher activity and good coincidence with the main peak of proteolipid eluted by this solvent mixture. Considerable variability in the shape of peaks was found in the elution profile of proteolipids. Good reproducibility could be obtained when two Sephadex LH-20 columns were run side by side using the same Sephadex LH-20 and proteolipids preparation. Despite variabilities, the specific coincidences of the peaks of radioactive acetylcholine and those of proteolipids showed excellent reproducibility.

Binding of cholinergic blocking agents to proteolipids

Dimethyl- ^{14}C -*D*-tubocurarine (10^{-8} — 2×10^{-8} moles in 0.5 ml methanol) was incubated with a solution of proteolipids in chloroform-methanol (2:1 v/v, 5 ml) and applied to the column as in the case of acetylcholine. Dimethyl- ^{14}C -*D*-tubocurarine, in this amount, had been completely bound to the column in the absence of proteolipid and no radioactivity

FIG. 5. Elution of proteolipids incubated with dimethyl- ^{14}C -*D*-tubocurarine at -60°C .

Proteolipids dissolved in chloroform-methanol (2:1 v/v) were incubated with 10^{-8} moles of dimethyl- ^{14}C -*D*-tubocurarine and applied to Sephadex LH-20 column. Subsequent procedures were the same as in Fig. 3.

was detected in the eluate with chloroform followed by chloroform-methanol (4:1 v/v). But in the presence of proteolipids the radioactivity of dimethyl-*d*-tubocurarine was recovered in the eluate with chloroform, showing excellent coincidence with the first peak of proteolipid (Fig. 5).

Higher concentration of dimethyl- ^{14}C -*d*-tubocurarine (5×10^{-8} moles in a similar incubation mixture) showed binding also with the proteolipids of the second peak as well as the first one. A similar reduced specificity of binding appeared in the case of ^3H -atropine even at a lower concentration. When ^3H -atropine sulfate (5×10^{-9} moles) was incubated and applied to Sephadex LH-20 column, this was bound to the proteolipids of both peaks of the chloroform fraction and to the peak of the chloroform-methanol fraction (Fig. 6).

FIG. 6. Elution of proteolipids incubated with ^3H -atropine at -60°C .

Proteolipids dissolved in chloroform-methanol (2:1 v/v) were incubated with 5×10^{-6} moles of ^3H -atropine and applied to Sephadex LH-20 column. Subsequent procedures were the same as in Fig. 3.

FIG. 7. Elution of proteolipids incubated with ^{14}C -decamethonium at -60°C .

Proteolipids dissolved in chloroform-methanol (2:1 v/v) were incubated with 2×10^{-6} moles of ^{14}C -decamethonium and applied to Sephadex LH-20 column. Subsequent procedures were the same as in Fig. 3.

Even if we reduced further the amounts of atropine sulfate to 0.5×10^{-9} moles, we got the same results. However, the binding of ^{14}C -acetylcholine to the proteolipids of the first peak of the chloroform elution was never observed even when a higher concentration of acetylcholine was used. ^{14}C -decamethonium gave the same results as in the case of dimethyl- ^{14}C -*d*-tubocurarine (Fig. 7).

Absence of acetylcholinesterase activity in cholinergic receptor fractions

In view of repeated proposals in the past that the anionic site of acetylcholinesterase might be the acetylcholine receptor (12, 13) we examined the proteolipid preparations for the activity of this enzyme. After extraction of proteolipids from brain powder with chloroform-methanol (2:1 v/v) the residue was separated by filtration and the organic solvent in the filtrate and residue was removed at subzero temperature (-68°C).

As shown on Table 1, the acetylcholinesterase activity was well maintained in the residue and it showed about 30% increase compared to the original brain powder. However, no enzymatic activity was observed in total lipid extract or in proteolipids precipitated by diethyl ether. Efforts were made to detect even a weak activity through sonication of these preparations or the addition of diluted aqueous methanol to bring the proteolipid solute into better contact with the substrate. No activity, however was detected.

TABLE 1. Acetylcholinesterase activity

Preparation	Hydrolysis of Acetylthiocholine (unit/mg protein/min)
Original brain powder	0.198 ± 0.003 (n=3) ^a
Residue after extraction at -68°C ^b	0.268 ± 0.034 (n=6)
Total lipid extract or proteolipids	none

Proteolipids from rat cerebral cortex were extracted with chloroform-methanol (2:1 v/v) at -68°C . The proteolipids and residue were separated by filtration and freed from organic solvent under high vacuum.

^aMean $\pm$ standard deviation.

^bRemoval of adherent solvent by molecular distillation at -68°C

Biphasic distribution experiments at ordinary temperature

For qualitative detection of binding, we had utilized coincidences in elution peaks of proteolipids with those of acetylcholine or cholinergic blocking agents and could get good resolution. But owing to the limited binding ability of Sephadex LH-20 column for these cholinergic agents, the data were not sufficiently reproducible quantitatively to yield discrete information concerning the binding. The more quantitative correlations were to be derived from changes induced by the presence of proteolipids fractions in the distribution ratio of cholinergic agents between two fluid phases in equilibrium. At the time of these experiments we had no appropriate biphasic system fluid at -60°C , so we turned to a system similar to the one used by G. Weber *et al.* (14), who measured by means of fluorescence in an organic-aqueous biphasic system at ordinary temperature, the increase in concentration of the cholinergic agent in the organic phase because of the presence of proteolipids in this

phase. We made use of the well known phase system that results when the solvent chloroform-methanol (2:1 v/v) is mixed with water.

Fig. 8 represents the concentration in the biphasic system of ^{14}C -acetylcholine, dimethyl- ^{14}C -*d*-tubocurarine, and ^{14}C -decamethonium as a function of the concentration of proteolipids introduced into this system. These three agents, initially added in the aqueous phase, were transferred to resulting organic phase in the presence of proteolipids in the organic phase.

The saturation values of binding of dimethyl-*d*-tubocurarine and of decamethonium were higher than that of acetylcholine and their initial slopes were much steeper. Even if the radioactive cholinergic agents were added initially to the organic solvent, almost all the radioactivity was transferred into the aqueous phase in the absence of added proteolipids. The resulting distribution between two phases was finally the same as when the radioactive material was added into the aqueous phase.

The first and second peaks of proteolipids separated by Sephadex LH-20 column chromatography at 60°C were used in the biphasic experiments. The binding of dimethyl- ^{14}C -*d*-tubocurarine occurred predominantly in solution containing the proteolipids of the first peak. In the binding experiments using Sephadex LH-20 column at -60°C , acetylcholine was exclusively bound to proteolipids of the second peak but in the biphasic analysis it was bound also to the proteolipids of the first peak although to a lesser extent (Table 2).

Proteolipids were successively precipitated from very diluted solutions (100 μg protein/ml) of total lipid extract below -60°C by precooled diethyl ether, two volumes of the latter to one of chloroform-methanol (2:1 v/v). The precipitation was very slow. The first precipitate obtained in total lipid extract contained per unit protein about 40% of the phos-

FIG. 8. The bindings of radioactive acetylcholine, dimethyltubocurarine, and decamethonium to proteolipids from rat cerebral cortex.

The proteolipids from rat cerebral cortex were prepared at -60°C . The dried ether precipitate was brought up to ice temperature and dissolved with chloroform-methanol (2:1 v/v). The solution was diluted by the same solvent to the proper concentration of proteolipids. The amount of the proteolipids in the reaction mixture was expressed by μg protein (absorption at 280 $m\mu$) per 3 ml of solvent mixture. Three milliliters of proteolipids solution were transferred to test tubes in an ice bath. To these tubes was added 1 ml of aqueous solution ($2 \times 10^{-5}\text{M}$) of ^{14}C -acetylcholine, ^{14}C -decamethonium or dimethyl- ^{14}C -*d*-tubocurarine. The tubes were shaken vigorously for 2 min using a Vortex mixer.

TABLE 2. Binding of ^{14}C -acetylcholine and dimethyl- ^{14}C -*d*-tubocurarine to the first and second peaks of proteolipids fractionated at -60°C

Fractions	^{14}C -ACh (n moles/mg protein)	^{14}C -DMTC
First peak	50.2	308.4
Second peak	83.5	10.4

Proteolipids were fractionated on Sephadex LH-20 column at -60°C . The first and second peaks eluted with pure chloroform were separated and diluted with chloroform-methanol (final ratio 2:1 v/v). The concentration of protein was adjusted to $100\text{ }\mu\text{g}/3\text{ ml}$. Each fraction was brought up to ice temperature and subjected to reaction with 1 ml of aqueous solution ($2 \times 10^{-6}\text{M}$) of ^{14}C -acetylcholine and dimethyl- ^{14}C -*d*-tubocurarine.

Values were the mean average of three experiments.

TABLE 3. Binding of ^{14}C -acetylcholine and dimethyl- ^{14}C -*d*-tubocurarine to the proteolipids which were precipitated repeatedly by diethyl ether

Times of ether precipitation	^{14}C -ACh (n moles/mg protein)	^{14}C -DMTC	Contents of phospholipid phosphorus (μ moles Pi/mg protein)
0*	54.5	131.0	4.9
1	68.4	148.0	2.0
2	66.5	155.0	1.3
3	54.0	143.0	1.2
4	54.1	140.0	1.2

Total lipid extract from rat cerebral cortex was subjected to repeated ether precipitation at -60°C . After bringing up to ice temperature it was submitted to reaction with ^{14}C -acetylcholine and dimethyl- ^{14}C -*d*-tubocurarine in biphasic system. Subsequent procedures were as in Fig. 8

*0: Total lipid extract

pholipid phosphorous of that in the total lipid extract. The second precipitate contained 26% and the third, and the fourth held to the same value. Biphasic analysis of binding showed that the amounts of ^{14}C -acetylcholine bound to the proteolipids scarcely changed in the separate preparations, despite the reduced phosphorous content. The same degree of constancy was observed in the binding of dimethyl- ^{14}C -*d*-tubocurarine by the successive preparations (Table 3).

In biphasic experiments, the binding of ^{14}C -acetylcholine to the proteolipids was inhibited by the presence of nonradioactive dimethyl-*d*-tubocurarine which was initially present in the organic phase. On the contrary, the binding of dimethyl- ^{14}C -*d*-tubocurarine to the proteolipids was not inhibited by the presence of nonradioactive acetylcholine in the organic phase, although it showed slight inhibition at a higher concentration (Fig. 9). The binding of acetylcholine was inhibited also by the presence of atropine sulfate, showing similar results as in the case of dimethyl-*d*-tubocurarine. A cholinergic receptor agonist such as nicotine or muscarine somewhat increased ^{14}C -acetylcholine binding at a lower

FIG. 9. Reciprocal inhibition to binding of radioactive acetylcholine and dimethyltubocurarine.

The ether precipitate dried completely by molecular distillation at -60°C was warmed to ice temperature and dissolved in the chloroform-methanol mixture (2:1 v/v) after which it was diluted to $45\text{ }\mu\text{g}$ proteolipid per ml. Two milliliters of this solution were precooled to ice temperature and then mixed with 1 ml of chloroform-methanol (2:1 v/v) containing non-radioactive acetylcholine or dimethyltubocurarine. Accordingly, the concentration of nonradioactive materials was expressed as a final concentration in the 3 ml of chloroform-methanol mixture. One milliliter of aqueous solution of ^{14}C -acetylcholine ($2 \times 10^{-8}\text{M}$) was added to the tubes containing nonradioactive dimethyltubocurarine and dimethyl- ^{14}C -tubocurarine ($2 \times 10^{-8}\text{M}$) was added to that containing nonradioactive acetylcholine. Subsequent procedures were the same as given in the footnote for Fig. 8.

FIG. 10. Effects of various agents on the binding of ^{14}C -acetylcholine to the proteolipids from rat cerebral cortex.

Dimethyl *d*-tubocurarine, atropine, nicotine and muscarine were mixed with $90\text{ }\mu\text{g}$ of proteolipids in 3 ml of chloroform-methanol (2:1 v/v). To these tubes 1 ml of aqueous solution of ^{14}C -acetylcholine was added. The subsequent operations were the same as given in the footnote for Fig. 8.

concentration. But at a higher concentration inhibition of ^{14}C -acetylcholine binding was observed (Fig. 10).

The pure phospholipids such as phosphatidyl serine and monophosphatidyl inositol showed affinity to acetylcholine as well as to cholinergic blocking agents (Fig. 11).

DISCUSSION

Proteolipids (1) are defined as the complex of protein and lipids extractable by a mixture of chloroform-methanol (2:1 v/v). But this solvent for lipids extraction is well known to modify the protein structure, at ordinary temperature, such as producing denaturation of membrane bound enzyme (8). It also induces dissociation of lipid components from the

FIG. 11. The bindings of radioactive acetylcholine (ACh), dimethyl-*d*-tubocurarine (DMTC) and decamethonium (C-10) to phospholipids from bovine brain.

Phospholipids were dissolved in the chloroform-methanol (2:1 v/v) and diluted to the desired concentrations. The subsequent operations were the same as given in the footnote for Fig. 8.

proteolipid complex.

The low temperature technique described in this paper, the rapid quenching of cerebral cortex at -196°C in liquid nitrogen and the following procedures carried out at or below -60°C , seem to maintain the proteolipids in better approximation to the *in vivo* structure, since at a lower temperature, dissociation of lipids components from proteolipids would be less probable in both energy and rate determined processes and proteins maintain their conformations.

De Robertis and his associates found that some fractions of proteolipids from the cerebral cortex did bind with cholinergic blocking agents (2, 3). Similar results were reported by them that acetylcholine bound with some of the proteolipids derived from the electric organ of electric fishes (7).

We attempted similar experiments entirely at low temperature using the proteolipids prepared from rat cerebral cortex. When ^{14}C -acetylcholine was incubated with diluted total lipid extract and applied on the Sephadex LH-20 column, good resolution was obtained in the elution peaks of proteolipids. The radioactive acetylcholine was observed in coincidence with the second peak eluted with pure chloroform and also in the main peak eluted with chloroform-methanol (4:1 v/v). This fact does not necessarily mean that more than two binding sites existed in the proteolipids of our preparation. The Sephadex LH-20 does not act merely as gel filtration but also as partition chromatography as instructed by Pharmacia. Some of the proteolipids together with acetylcholine were tightly bound to Sephadex LH-20 depending on the binding ability of this column and were eluted with the more polar solvent mixture of chloroform-methanol (4:1 v/v).

Levinson and Keynes claimed that the coincidences of peaks of proteolipids and cholinergic agents from Sephadex LH-20 column might be misleading, because they may be

caused by solvent front effects due to the increased polarity of the elution solvent mixture (15). The fact that we obtained the same results by the delayed application of ^{14}C -acetylcholine and proteolipids as well as when we applied the mixture of ^{14}C -acetylcholine and proteolipids gave a strong proof that the coincidences of peaks were not artificial. Furthermore, by using a column longer than the one they used, we could not detect any solvent front on monitoring the change of absorbance at 254 m μ .

In the previous reports, cholinergic blocking agents were usually used to label the cholinergic receptors. But the question naturally arises as to whether or not the acetylcholine receptor also binds the cholinergic blocking agents. It is evident that the specificities of binding of ^{14}C -acetylcholine and of dimethyl- ^{14}C -*d*-tubocurarine were directed toward different proteolipid fractions, the second and first peak respectively, suggesting that the receptor fraction for acetylcholine might be different from that for cholinergic blocking agents. Such discrimination of receptor fractions into two types had not been reported in previous works with extracts of brain tissue or of electroplax and have been made possible through proteolipids fractions prepared at subzero temperatures. Such preparations would be expected to maintain more nearly intact the *in vivo* structure with its specificities than those prepared at ordinary temperatures.

The repeated proposal that the anionic site of acetylcholinesterase might be the acetylcholine receptor (12, 13) was reexamined in this paper, because our experimental conditions were very favorable for this purpose. At subzero temperature, the activity of acetylcholinesterase was well maintained in the tissue residue which would be completely denaturated in a similar organic solvent at the usual temperature. On the contrary, we could not detect any activities in extracted proteolipids. These results are in good agreement with those of Azcurra and De Robertis (16) who found no changes in the binding of methyl- ^{14}C -hexamethonium to preparations of isolated electroplax membrane that had different activities of acetylcholinesterase. This view gains support also from the report presented by Miledi *et al* (17) that the binding of α -bungarotoxin to cholinergic receptor was decreased in the presence of *d*-tubocurarine but was unaffected by acetylcholinesterase inhibitors.

The receptor proteolipids for acetylcholine and acetylcholinesterase may be localized very closely *in vivo* on the postsynaptic membrane. After acetylcholine is bound to its receptor fraction and then displays its physiological role as neurotransmitter, the following process will be the translocation of acetylcholine to the site on acetylcholinesterase. In our experiment, the acetylcholinesterase activity in the residue was increased somewhat 30% after removal of the proteolipids from the tissue. One possibility is that the proteolipids may act as an intrinsic inhibitor of acetylcholinesterase, although we did not estimate the enzyme activity of residue when it was reconstituted with proteolipids. Once acetylcholine bound with the proteolipids, their inhibitory action would be diminished causing an increase in this enzyme activity necessary for the hydrolysis of acetylcholine. The receptor proteolipid could then have a regulatory function. The importance of cholinergic receptor protein as a regulatory protein was extensively studied and discussed by Changeux *et al* (18–20) in *Electrophorus electric organ*.

As a conclusion, the extraction and fractionation of proteolipids from rat cerebral cortex at subzero rather than ordinary temperatures yielded preparations apparently in closer correspondence to physiological states. These preparations strongly indicate that the acetylcholine receptor fraction and that for cholinergic blocking agents are sharply differentiated and acetylcholinesterase was not present in either type of receptor fractions.

The existence of the two differentiated receptor proteolipid fractions persisted in the organic solvent despite warming to ordinary temperatures (first to 0°C, and then warming to room temperature after addition of water) as demonstrated in biphasic equilibria. These specificities unobserved in previous work at ordinary temperature suggest that extraction of proteolipids at low and ordinary temperatures lead to different structures in solution.

To account for the loss in specificity of acetylcholine binding at ordinary temperature, the following mechanism is proposed. A change in the surface properties of proteolipids in the presence of water may be expected to have some resemblance to the inversion of micellar phases as discussed by Colacicco *et al* (21) in their study of the surface properties of the apoprotein of proteolipids. In water, the hydrophobic structures become localized as the inner phase of the micelle while the polar structures come to the surface. In the biphasic analysis, shaking the proteolipids with a mixture of chloroform-methanol and water would induce polar groups previously within the inner structure to come to the surface, remaining there because of the water in the organic phase. The polar groups may then bind acetylcholine to the proteolipid fraction that at low temperature in the absence of water had remained unbound.

Although successive precipitation of proteolipids with diethyl ether removed more than 70% of phospholipids from total lipid extract, the binding abilities of proteolipids for acetylcholine and dimethyl-*d*-tubocurarine were not changed. Free and loosely bound phospholipids seems to be removed by ether precipitation. Phospholipids are known to be components of the proteolipids. Actually, pure phospholipids such as phosphatidyl inositol and phosphatidyl serine showed affinity to acetylcholine and cholinergic blocking agents but with much decreased specificities. Therefore, although the tightly bound phospholipids may act as a part of binding sites for cholinergic substances, the protein moiety of proteolipids seems to be of primary importance in determining the specificities of receptor fractions.

If we provisionally accept the receptor proteolipids as models for the physiological cholinergic receptors, because of the distinctly different specificities, we conclude that the receptor for acetylcholine and blockers need not be the same so that direct molecular competition or displacement may not be a requirement for blocking the functional activity of nervous transmission. In this connection, the disparity observed in the reciprocal inhibitions of acetylcholine and dimethyl-*d*-tubocurarine in their binding to proteolipids seems important. Physiologically, they would be localized very closely on the postsynaptic membrane and once the dimethyl-*d*-tubocurarine bind to its own receptor fraction, some conformational changes may take place leading to the inhibition of the acetylcholine receptor fraction to bind acetylcholine. The finding that the binding of acetylcholine to proteolipids,

in biphasic experiments, was inhibited both by dimethyl-*d*-tubocurarine and atropine shows that both nicotinic and muscarinic types of cholinergic receptor fraction were involved in our preparation. Recently, Barrantes *et al.* have reported the separation of nicotinic and muscarinic hydrophobic protein from skeletal muscle and smooth intestinal muscle respectively using Sephadex LH-20 column (22). Both types of hydrophobic protein were recovered in the second peak eluted with pure chloroform, the similar fraction that bound acetylcholine in our experiment.

For further discrimination of the cholinergic receptor mechanism, we propose the use of labeled nicotine and muscarine in addition to acetylcholine and cholinergic blocking agents. The low temperature method described in this paper would be an aid to maintain the stereospecificity of the binding site to these agonists and antagonists.

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