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ACETYLCHOLINE-BINDING SUBSTANCE EXTRACTED BY USING ORGANIC SOLVENT AND ACETYLCHOLINE RECEPTOR OF ELECTRIC ORGAN OF *NARKE JAPONICA*

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SUMMARY

1. Proteolipid was extracted from the electric organ of *Narke japonica* by using chloroform/methanol (2 : 1, v/v). This extract was separated into acetylcholine-binding and non-binding substances by column chromatography. However, acetylcholine-binding substances did not show the characteristic properties of protein.

2. The membrane fragments of the electric organ were separated into three main parts by sucrose density gradient centrifugation. From the heaviest, the fractions were acetylcholine receptor rich, ATPase rich, and acetylcholinesterase rich.

3. The membrane fraction having acetylcholine receptor showed the excitability, the increase of Na^+ permeability by the application of cholinergic agonists. However, the acetylcholine binding substance extracted by the organic solvent was richer in the lighter fraction. This substance differed from the true acetylcholine receptor.

INTRODUCTION

De Robertis et al. [1–7] extracted a proteolipid from nerve endings by using chloroform/methanol (2 : 1, v/v) and examined its binding to transmitters such as acetylcholine in column chromatography, using Sephadex LH-20. Some peaks of the eluted proteolipid coincided with the radioactivity of the transmitter when the proteolipid was passed through the column with the transmitter. They obtained a large amount of the substance, which they termed acetylcholine receptor proteolipid, from the electric organ of *Torpedo* [4] or *Electrophorus* [6]. This substance which will be referred to as acetylcholine-binding substance in the text, in order to distinguish from the true acetylcholine receptor, was believed to be a true acetylcholine receptor from the following experiments. (1) The binding of acetylcholine was completely inhibited in the presence of α-bungarotoxin [8]. (2) The conformational change was caused by atropine [9]. (3) When this substance was incorporated into a black lipid membrane, a conductance change was caused by acetylcholine or its analogues [10, 11].

This isolation procedure was very simple compared with the preparation of the acetylcholine receptor reported by other researchers [12, 13]. In the latter case the substance of the electric organ was solubilized with a detergent such as Triton X-100 or deoxycholate, and separated by affinity chromatography. Moreover, the lipid-soluble substance is convenient for the study of the reconstitution of membrane vesicles containing the receptor or incorporation into black lipid membranes, etc.

However, in spite of the extensive study of De Robertis et al. [1–7], many problems have been pointed out. (1) From the sample not containing protein, the similar elution peak of acetylcholine was observed [14]. (2) The acetylcholine receptor bound with a specific ligand was not extracted with 2 : 1 chloroform/methanol (Sugiyama, H., private communication). (3) Even when the acetylcholinesterase was incorporated into black lipid membrane, the similar conductance change elicited by acetylcholine was observed [15].

Under these situations we examined the acetylcholine receptor proteolipid from different standpoints. (1) Does the peak of proteolipid coincide with that of acetylcholine by the column chromatography of Sephadex LH-20 according to De Robertis? What is the molecular weight and the purity of the proteolipid? (2) The membrane fragments of the electric organ can be separated into many fractions by sucrose density gradient centrifugation. What is the relationship between the acetylcholine receptor content, the excitability, and the acetylcholine-binding ability of the extracted proteolipid?

As the result, the acetylcholine-receptor proteolipid extracted by organic solvent had an acetylcholine binding ability comparable with the acetylcholine receptor, but was a different substance from the acetylcholine receptor essential for the nerve transmission.

MATERIALS AND METHODS

Extraction and chromatography of acetylcholine-binding substance. Extraction of the total lipid and the binding of acetylcholine to this material were examined according to the method of De Robertis et al. [4, 6]. The total lipid was extracted from *Narke japonica* (electric fish similar to *Torpedo*) by chloroform/methanol (2 : 1, v/v) and concentrated. Before the column chromatography, this total lipid extract was equilibrated for 20 min with about 0.5 nmol of acetyl[^{14}C]choline (13.9 μCi/mg) solubilized in 0.01 M methanolic HCl.

Sephadex column was prepared according to Soto et al. [2] with slight modification. The gel swollen with chloroform was packed in a column (25 $\times$ 45, SR-25/45 Pharmacia Fine Chemicals, Uppsala, Sweden). The bed height was about 22 cm. The elution was first made with 200 ml of chloroform, followed by mixtures of chloroform/methanol. Fractions of about 5 ml were collected. The ethanol contained in chloroform as a stabilizer was removed by a distillation before use. Protein content of each fraction was estimated by ultraviolet absorption at 280 nm. For the determination of the radioactivity, 0.5 ml of sample was dried in a vial tube, 5 ml of toluene/Triton scintillator (1420 ml toluene, 500 ml Triton X-100, 8 g PPO, 0.1 g POPOP) was added, and the counting was made in a Horiba liquid scintillation spectrometer. The ultraviolet absorption spectrum was measured by a Hitachi 124 with a reference of the same solvent.

Separation of membrane fragments by sucrose density gradient. Fractionation was made according to Cohen et al. [16]. A sucrose density gradient was formed by layering from bottom to top 4 ml each of 1.6, 1.2, 0.8 M sucrose. The tubes were centrifuged in a RPS 25-1 rotor of a Hitachi 65P ultracentrifuge for 5 h at 4 °C at 64 000 $\times g$. Fractions of 1.0 ml were collected.

Measurements. Proteins were estimated by the method of Lowry et al. [17] using bovine serum albumin as the standard. Acetylcholinesterase activity was assayed with acetylthiocholine as substrate by the method of Ellman et al. [18]. ATPase reaction was made in 2 mM ATP, 1 mM $MgCl_2$, 5 mM KCl, 58 mM NaCl, 0.1 mM EDTA, 0.1 mM Tris/maleate (pH 7.0) and the liberated inorganic phosphate was determined by the method of Taussky and Shorr [19] after stopping the reaction with trichloroacetic acid.

The cholinergic receptor protein present in the membrane fragments was labelled by Erabutoxin b from the venom of *Laticauda semifasciata* iodinated and purified by the method of Sato and Tamiya [20] with a slight modification (1.5 Ci/mol). 50 μl of toxin (175 pmol) was mixed with 3 ml of membrane fragments and left standing for 1 h at room temperature. The mixture was diluted to 10 ml, and centrifuged at 77 000 $\times g$ for 30 min. The pellet was mixed with 20 ml of toluene/Triton scintillator and the radioactivity was determined.

Excitability of the membrane fragments was determined as described in the previous paper [21] by using Na^+ permeability change caused by cholinergic agonist. The excitability was defined as $\tau_0/\tau - 1$, where τ_0 and τ are the half-decay time of the remaining $^{22}Na^+$ in the absence and in the presence of 0.1 mM carbamylcholine, respectively.

Electrophoresis in sodium dodecyl sulfate was carried out by the method of Fairbanks et al. [22]. As the indicator, Coomassie Brilliant Blue R was used.

Determination of acetylcholine binding to total lipid extract of separated membrane fragments by a partition method. The binding of acetylcholine to the total lipid extract was measured by a partition method similar to the method described by Weber et al. [7]. Separated membrane fractions were lyophilized after removal of the sucrose. The total lipid was extracted by 5 ml of chloroform/methanol/(2 : 1, v/v) for 5 min at room temperature. After filtration, the residues were re-extracted with 1 ml of the solvent. 1 ml of distilled water was then added to the extract. After a vigorous shaking, the mixture was centrifuged at 1000 $\times g$ for 10 min, and the upper phase was discarded. Further, 1 ml of chloroform/methanol/water (3 : 47 : 48, v/v) mixture was added and the same treatment as above was made twice. The resultant lower phase was regraded as the total lipid extract.

To each 1 ml of the total lipid extract, from 6 to 30 nmol of acetyl[^{14}C]choline was added. After standing for 20 min, 1 ml of a chloroform/methanol/water (3 : 47 : 48, v/v) mixture was added to the samples, followed by vigorous shaking. The samples were left standing for 2 h and then centrifuged at 1000 $\times g$ for 10 min. 0.5 ml of each phase was collected in a vial tube and the radioactivity was determined after drying, as before.

As a control the partition coefficient of free acetylcholine was determined without lipid by the same procedure. The amount of bound acetylcholine to the total lipid extract was determined from the acetylcholine content found in the lower phase minus free acetylcholine content calculated by using the partition coefficient.

Materials. The electric fish, *N. japonica*, were obtained from the marine laboratory of the University of Tokyo at Misaki, Kanagawa. Erabutoxin b was a gift from Professor N. Tamiya of Tohoku University. Acetyl[1-^{14}C]choline iodide was purchased from New England Nuclear, U.S.A., and Na^{131}I solution from Commissariat à l'Energie Atomic, France. Other reagents were of analytical grade.

RESULTS

(1) *Chromatography of the total lipid extract by Sephadex LH-20*

In Fig. 1, a typical chromatogram of the total lipid extract was shown. On the distribution of protein, one peak was found at 50 ml of the elution volume, several peaks at 120–200 ml, and two peaks at 300–360 ml. Acetylcholine peaks corresponded only to the several protein peaks at 120–200 ml. This experiment was repeated many times, but the acetylcholine peak did not correspond to one protein peak. This result differed from that reported by De Robertis et al. [4, 6].

Fig. 2 shows the result obtained by using stabilized chloroform containing 1 % ethanol in the process of the elution. In this case, few peaks of protein were found at 60–80 ml to which acetylcholine peaks also corresponded. The peak position was near the original papers [4, 6], but another peak of protein was found at 15 : 1 chloroform/methanol.

When the sample solution was concentrated to 2 ml and passed by the column with the same procedure to Fig. 2, a very similar protein distribution was obtained as shown in Fig. 3. However, the acetylcholine was eluted after chloroform/methanol (15 : 1, v/v).

In order to check the protein distribution at 120–200 ml in Fig. 1, the polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried

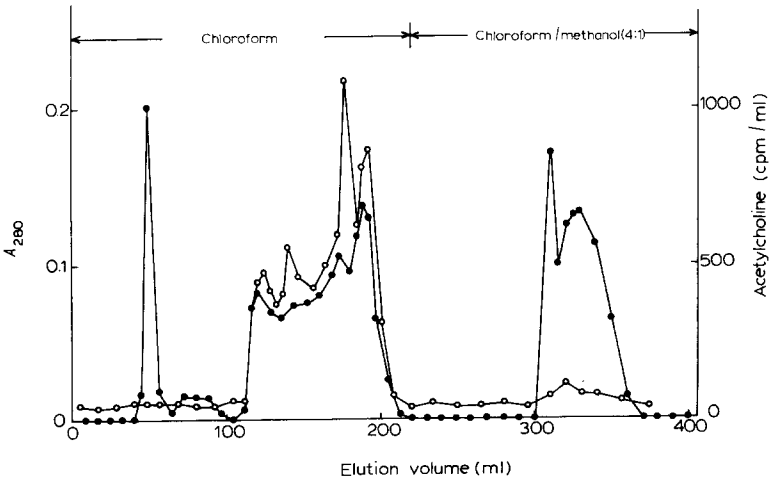

Fig. 1. Chromatogram of total lipid extract (1). 5 ml of sample was loaded on Sephadex LH-20 column and eluted by using distilled chloroform. ●, absorbance at 280 nm; ○, radioactivity of acetylcholine.

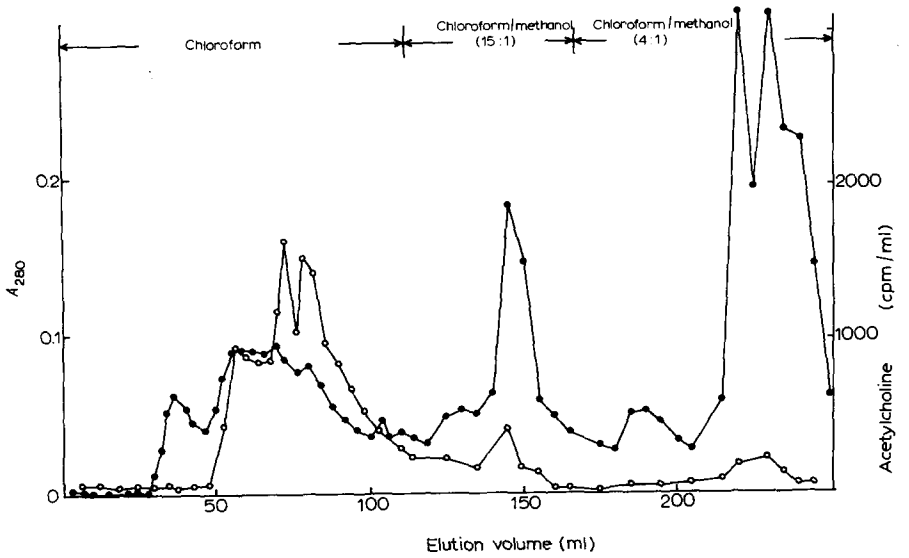

Fig. 2. Chromatogram of total lipid extract (2). 5 ml of sample was eluted by using stabilized chloroform containing 1 % ethanol. The symbols are the same as Fig. 1.

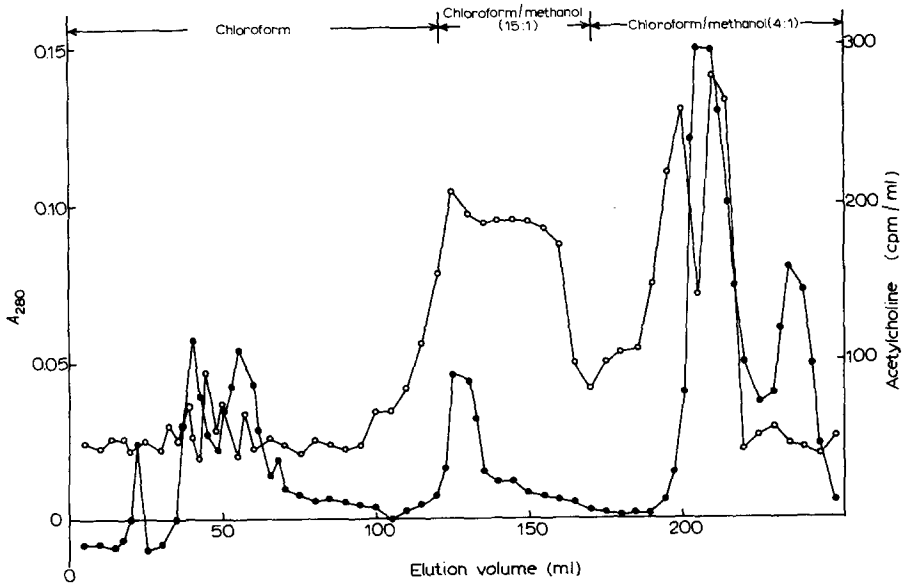

Fig. 3. Chromatogram of total lipid extract (3). 2 ml of sample was eluted by the same procedure of Fig. 2. The symbols are the same as Fig. 1.

out on this sample. Although a sufficient amount of protein had been expected to be present from the ultraviolet absorption at 280 nm, a protein-like band was not observed. Only an opaquely stained ellipsoidal part was observed near the marker. Accordingly, the ultraviolet absorption spectrum of this fraction was measured. Each fraction showed the absorption maximum at 290 nm and another peak at 250 nm. However, characteristic peaks of protein found at 278 nm and 240 nm were not observed. When the Folin method was applied [17], the sample became slightly colored, but the measurement was not made because of the turbidity.

(2) *Fractionation of membrane fragments by sucrose density gradient centrifugation*

After a density gradient centrifugation, membrane fragments were separated into three main parts. The heaviest fraction (between 1.6 and 1.2 M sucrose) was acetylcholine receptor rich and had slight ATPase, but no acetylcholinesterase. The middle fraction (between 1.2 and 0.8 M sucrose) also contained acetylcholine receptor, and had high ATPase, but contained no acetylcholinesterase. The lightest fraction (lighter than 0.8 M sucrose) showed only acetylcholinesterase activity and had neither acetylcholine receptor nor ATPase. This pattern was very similar to that reported by Cohen et al. [16] in the case of *Torpedo marmorata*. The characteristic properties of these membrane fractions were summarized in Table I.

Since there was a definite separation of membrane fragments, the protein distribution was examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The heaviest and the middle fractions had the similar distribution pattern and the lightest fraction was essentially a different pattern. In all fractions a large peak of protein having a molecular weight of about 100 000 was seen. In the lightest fraction, ATPase was not observed at all, so most of this peak might be a structural protein rather than ATPase. In the lightest fraction, a large peak of about 40 000 dalton was observed.

Excitability of these fraction was examined by using the permeability change for $^{22}Na^{+}$ caused by carbamylcholine. The result was also shown in Table I. The heaviest fraction had the highest excitability and the middle fraction was the same. But the lightest fraction did not have excitability. Namely, the excitability was only found in the membrane fragments containing acetylcholine receptor and separated from the acetylcholinesterase activity. This result was consistent with the observation of the *Torpedo* membrane [16, 23], but different from the *Electrophorus* membrane [24].

With reference to the experiment made in the previous section, acetylcholine binding of the total lipid extract was examined in each fraction by using a partition method. By increasing the concentration of acetylcholine, the amounts of acetylcholine found in the chloroform and in the water phase were determined. Lineweaver-Burk plot of acetylcholine found on the chloroform phase was almost linear in this concentration range of acetylcholine. The amount of the sites and the dissociation constant for acetylcholine were determined (Table I). Dissociation constant differed from fraction to fraction, but the number of sites was very rich in the lightest fraction. This result shows that the acetylcholine-binding properties of the lipid extract had no direct relation to the acetylcholine receptor content determined by the toxin binding. However, it was shown that the acetylcholine-binding properties found in the experiment described in the previous section was not an artifact and that the lipid extract had a comparable acetylcholine-binding ability to the true acetylcholine receptor.

TABLE I

CHARACTERISTIC PROPERTIES OF SEPARATED MEMBRANE FRAGMENTS

Name	Sucrose concentration (M)	Acetylcholin-esterase (pmol/mg*)	ATPase (μmol P_i/h per mg*)	Acetylcholine receptor (pmol/mg*)	$^{22}Na^+$ flux		Acetylcholine binding of lipid extract	
					τ_0 (min)	Excit-ability ($\tau_0/\tau - 1$)	No. of sites (nmol/mg**)	K_d (μM)
Acetylcholine receptor rich	1.6–1.2	0.61	5.5	12.2	3	1.0	0.21	3.0
ATPase rich	1.2–0.8	0.36	16.0	5.0	4.5	0.8	0.61	3.4
Acetylcholinesterase rich	0.8–0.0	6.4	0.0	2.4	10.0	0.0	1.3	17
Crude fragments		1.22	4.1	2.6				
Total tissue							1.2	7.8

* Expressed by mg protein.

** Expressed by mg dry weight.

DISCUSSION

At first this experiment was initiated with the intention to confirm the experimental result of De Robertis et al. [1–7] by using the electric tissue of *N. japonica*. However, as described in Results, we could not confirm all results. As a result, acetylcholine-binding substance extracted by organic solvent had a acetylcholine-binding ability, but differed from the true acetylcholine receptor.

Similar experiments have been reported by Levinson and Keynes [14]. In that report, they concluded that the binding of acetylcholine to the lipid extract demonstrated by Sephadex LH-20 chromatography was an artifact due to the operation of column. The basis of that conclusion was as follows. (1) When the chromatography was carried out in the same condition as reported by De Robertis, the peaks of proteolipid and acetylcholine were found always at the position corresponding to the bed volume. This was an artifact caused by the fact that too much sample was loaded on a column. Then, when a small volume of sample was loaded, acetylcholine could not be eluted in the chloroform phase. (2) From the sample not containing protein or acetylcholine receptor, the similar result was obtained, etc.

Our result partially confirmed this experiment. Proteolipid and acetylcholine were eluted at the position of the bed volume when the chloroform containing ethanol was used as shown in Fig. 2. However, when the chloroform not containing ethanol was used, proteolipid and acetylcholine were eluted at the position corresponding about twice the bed volume as shown in Fig. 1. In our experiment, the peak of acetylcholine was not single in any case. This was the different result from De Robertis et al. [4, 6]. However, this substance possessed acetylcholine-binding ability. From the polyacrylamide electrophoresis, and the ultraviolet absorption spectrum, this material did not show the characteristic properties of a protein. However, we did not make a further investigation on this point.

From the experiment carried out by a chromatography the relation between this substance and the true acetylcholine receptor was not clear. Then the membrane fragment was fractionated by sucrose density gradient and the relations among acetylcholine receptor, the excitability, and the acetylcholine binding ability were examined. As shown in Table I, acetylcholine receptor estimated by toxin binding ability and the excitability showed a good agreement. However, acetylcholine-binding substance extracted by organic solvent was present in all fractions and was especially rich in the acetylcholinesterase-containing fraction. Moreover, in the total tissue almost the same concentration of acetylcholine-binding site was present. From these experiments, concerning acetylcholine-binding proteolipid extracted by the method of De Robertis et al., the following conclusion could be made. (1) This is an acetylcholine-binding substance, (2) the properties of protein are lacking, and (3) this substance has no direct relationship with the true acetylcholine receptor.

As the properties of this substance, the dissociation constant obtained in our experiment, $3.0 \cdot 10^{-6}$ and $1.7 \cdot 10^{-5}$ M was similar to the values reported by De Plazas and De Robertis [8] as two types of sites, $1 \cdot 10^{-7}$ and $1 \cdot 10^{-5}$ M. The number of sites was about $6 \cdot 10^{-10}$ mol/mg dry tissue. This value is about 100 times higher than the true acetylcholine receptor sites obtained in Table I, although the amount of the true acetylcholine receptor might be underestimated. The function and the localization of this substance are still unknown. On the excitable membrane

there must exist many kinds of acetylcholine-recognizing sites in addition to acetylcholinesterase or acetylcholine receptor, for example, acetylcholine-binding sites related to release, transport, storage or synthesis. The investigation of such acetylcholine-binding substances would lead to an interesting result [25].

As far as the fractionation of membrane fragments is concerned, three types of membrane fragments having acetylcholine receptor, ATPase and acetylcholinesterase were separated by sucrose density gradient centrifugation. The result was very similar to the case of *T. marmorata* reported by Cohen et al. [16] and that of *Torpedo californica* reported by Duguid and Raftery [23], but a much better separation of acetylcholine receptor and ATPase was obtained. Electrophoresis pattern was also similar to the case of *T. californica* [23]. In crude membrane homogenate, the contents of acetylcholine receptor and acetylcholinesterase were about 1–3 and 0.5–2 pmol/mg, respectively, and in the fractionated membrane fragments, these values were 10–20 and 2–10 pmol/mg, respectively. From these results, the membrane construction of the electric organ of *Narke* must be similar to that of the *Torpedo*. However, the content of acetylcholine receptor and acetylcholinesterase was about 10 times smaller than the reported values of *Torpedo* [16].

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REFERENCES

1 De Robertis, E. (1971) Science 171, 963–971
2 Soto, E. F., Pasquini, J. M., Placido, R. and La Torre, J. L. (1969) J. Chromatogr. 41, 400–409
3 De Robertis, E., Fiszer, S., Pasquini, J. M. and Soto, E. F. (1969) J. Neurobiol. 1, 41–52
4 La Torre, J. L., Lunt, G. S. and De Robertis, E. (1970) Proc. Natl. Acad. Sci. U.S. 65, 716–720
5 De Robertis, E. and De Plazas, S. F. (1970) Biochim. Biophys. Acta 219, 388–397
6 De Robertis, E., Lunt, G. S. and La Torre, J. L. (1971) Mol. Pharmacol. 7, 97–103
7 Weber, G., Borris, D. P., De Robertis, E., Barrantes, F. J., La Torre, J. L. and Llorente De Carlin, M. C. (1971) Mol. Pharmacol. 7, 530–537
8 De Plazas, S. F. and De Robertis, E. (1972) Biochim. Biophys. Acta 274, 258–265
9 Rodriguez, J. G., La Torre, J. L. and De Robertis, E. (1970) Mol. Pharmacol. 6, 122–127
10 Parisi, M., Reader, T. A. and De Robertis, E. (1972) J. Gen. Physiol. 60, 454–470
11 Reader, T. A., Parisi, M. and De Robertis, E. (1973) Biochem. Biophys. Res. Commun. 53, 10–17
12 Karlsson, E., Heilbronn, E. and Widlund, L. (1972) FEBS Lett. 28, 107–111
13 Meunier, J. C., Sealock, R., Olsen, R. and Changeux, J. P. (1974) Eur. J. Biochem. 45, 371–394
14 Levinson, S. R. and Keynes, R. D. (1972) Biochim. Biophys. Acta 288, 241–247
15 Jain, M. K. (1974) Arch. Biochem. Biophys. 164, 20–29
16 Cohen, J. B., Weber, M., Huchet, M. and Changeux, J. P. (1972) FEBS Lett. 26, 43–47
17 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
18 Ellman, G. L., Courtney, K. D., Andres, Jr, V. and Featherstone, R. M. (1961) Biochem. Pharmacol. 7, 88–95
19 Taussky, H. H. and Shorr, E. (1953) J. Biol. Chem. 202, 675–685
20 Sato, S. and Tamiya, N. (1970) J. Biochem. Tokyo 68, 867–872

21 Kasai, M. and Changeux, J. P. (1971) J. Membrane Biol. 6, 1–23
22 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617
23 Duguid, J. R. and Raftery, M. A. (1973) Biochemistry 12, 3593–3597
24 Kasai, M. and Changeux, J. P. (1971) J. Membrane Biol. 6, 24–57
25 Izumi, F. and Freed, S. (1974) FEBS Lett. 41, 151–155