SYNTHESIS AND PHARMACOLOGICAL ACTIVITY ON *ELECTROPHORUS ELECTRICUS* ELECTROPLAQUE OF PHOTOAFFINITY LABELLING DERIVATIVES OF THE NON-COMPETITIVE BLOCKERS DI- AND TRI-METHISOQUIN

Gilles WAKSMAN, Robert OSWALD+, Jean-Pierre CHANGEUX+ and Bernard P. ROQUES

Département de Chimie Organique, ERA 613 (CNRS), UER des Sciences Pharmaceutiques et Biologiques, 4 avenue de l'Observatoire, 75270 Paris Cedex 06 and *+Neurobiologie Moléculaire et Laboratoire associé au Centre National de la Recherche Scientifique, Interactions Moléculaires et Cellulaires, Institut Pasteur, 25 rue du Docteur Roux, 75015 Paris, France*

Received 17 December 1979

1. Introduction

The physiological response of the neuromuscular junction, or of the fish electroplaque, to acetylcholine (ACh) is blocked by two distinct classes of pharmacological agents [1]. The competitive antagonists such as d-tubocurarine or flaxedil bind at the level of (or close to) the receptor site for acetylcholine where they block the effect of the agonists possibly by steric hindrance. This site is also labelled by snake venom α-toxins [2] and the covalent affinity labelling reagents: TDF [3,4]; MPTA [5]; bromoacetylcholine [6]; DAPA [7]. Another rather heterogeneous group of compounds includes the aminated local anesthetics [1,8] and the frog toxin, histrionicotoxin [9,10], which block the response to ACh in a non-competitive manner. In vitro studies carried out with radioactive [8,11–13] or fluorescent [12,14–18] ligands have shown that these non-competitive blockers bind to a class of sites distinct from the ACh-receptor site [11, 12]. Moreover, at equilibrium [12] or after rapid mixing [14,17,18] the two classes of sites have been shown to interact in an allosteric manner. Biophysical experiments suggest that these non-competitive blockers bind directly to the ion gate opened by ACh [19,20], although indirect effects are also possible [21]. In any case, compounds of this series behave as potential labels of the ion translocating device, or ionophore, regulated by ACh.

In an attempt to label the site for non-competitive blockers on *Torpedo* receptor-rich membranes, a photoaffinity labelling derivative of the local anesthetic procaine (procaine amide azide) was synthetized [22]. This compound covalently labels [22] the 43 000 M_r polypeptide recently discovered in the ACh receptor-rich membranes [23]. However, removal of this 43 000 M_r polypeptide by pH 11 treatment does not interfere with the reversible binding of the radioactive local anesthetic [^{14}C]meproadifen, and with the allosteric interaction between ACh receptor site and local anesthetic binding site [24] indicating that the 43 000 M_r polypeptide and, of course, the site labelled by procaine amide azide, are not the site of the pharmacological action of the non-competitive blockers.

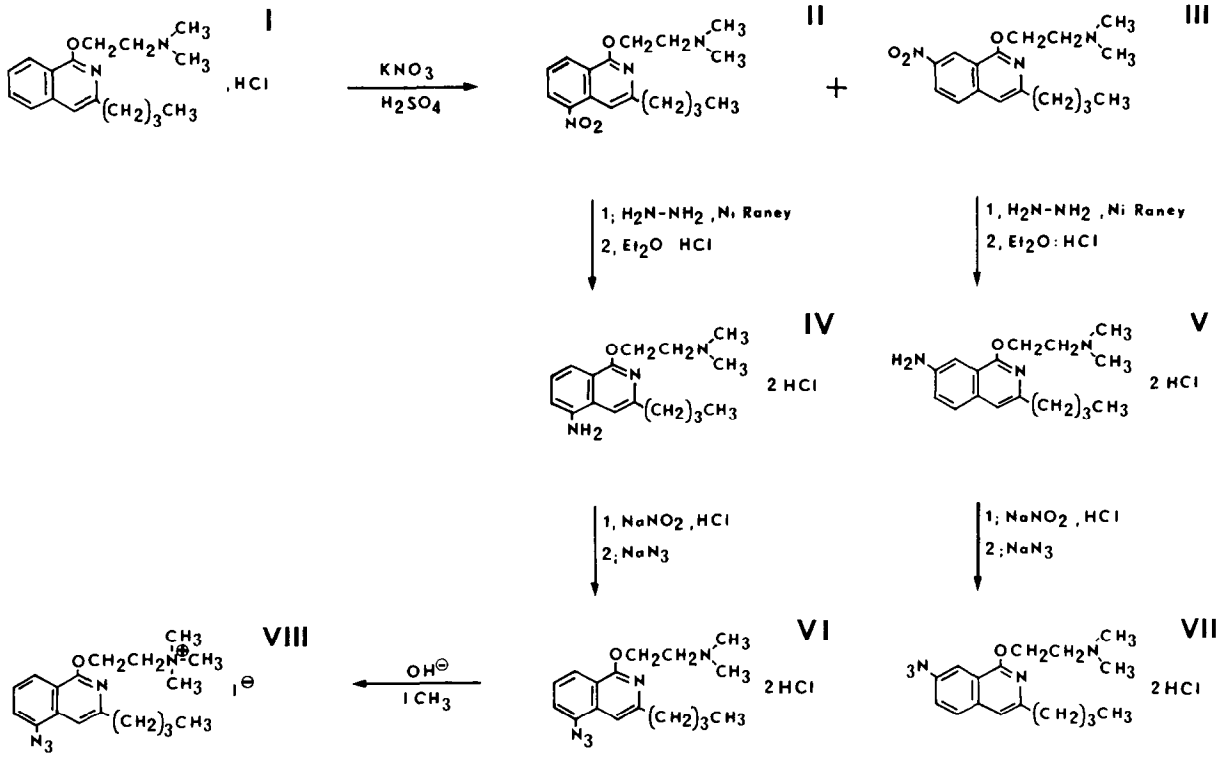
Here we describe the synthesis of photoaffinity derivatives of two potent non-competitive blockers of the electroplaque synapse: dimethisoquin [11,12] and trimethisoquin [13]. These compounds 5- and 7-azido dimethisoquin and 5-azido trimethisoquin behave as irreversible blockers of the response of *Electrophorus electricus* electroplaque to cholinergic agonists. In [25] the selective labelling by [^{3}H]azido 5-trimethisoquin of some of the polypeptide chains present in *Torpedo* ACh receptor-rich membranes is described.

Abbreviations: ACh, acetylcholine; TDF, *p*-trimethylammonium benzene diazonium fluoroborate; MPTA, 4-(*N*-maleimido) phenyltrimethylammonium; DAPA, bis(3-azidopyridinium)-1,10-decane perchlorate; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; HMDS, hexamethyldisiloxane

2. Material and methods

2.1. *Synthesis*

The synthesis of the azide compounds was performed as shown in scheme 1.



3-n-butyl-5-nitro-1-[2-(dimethylamino)ethoxy] isoquinoline, II
and
3-n-butyl-7-nitro-1-[2-(dimethylamino)ethoxy] isoquinoline, III

Potassium nitrate, 656 mg (6.48 mmol) was added at 0°C to a solution of 2 g (6.48 mmol) dimethisoquin (I) in 8 ml sulfuric acid (d = 1.84). The mixture was stirred for 48 h at room temperature. The resulting solution was poured onto ice, treated with 12 N NaOH to pH 11 and extracted 3 times with 50 ml ethyl acetate. The organic phases were dried on Na_2SO_4 and evaporated to dryness in vacuo giving 2.1 g residual oil. The oil was purified by chromatography on silica gel column using diethyloxide as eluent.

The oily 7-nitro derivative (III), 346 mg, was eluted first.
Yield: 17%. R_F = 0.55 in Et_2O:MeOH (1:1) on silica gel plates.
Anal. calc. for $C_{17}H_{23}N_3O$: C, 64.33; H, 7.3; N, 13.24.
Found: C, 64.12; H, 7.38; N, 13.15.
NMR (aromatic part) δ ppm: H_4, 7.17; H_6, 7.78; H_5, 8.26; H_8, 8.88.

The oily 5-nitro derivative (II), 840 mg, was then eluted.
Yield: 41%, R_F = 0.40 in Et_2O:MeOH (1:1).
Anal. found: C, 64.52; H, 7.12; N, 13.35.
NMR (aromatic part) δ ppm: H_7, 7.38; H_4, 7.55; H_6, 8.25; H_8, 8.4.

3-n-butyl-5-amino-1-[2-(dimethylamino) ethoxy] isoquinoline dihydrochloride, IV
and
3-n-butyl-7-amino-1-[2-(dimethylamino) ethoxy] isoquinoline dihydrochloride, V

The nitro derivatives II and III were reduced by the Raney-nickel hydrazine hydrate method [26]. Raney-nickel was cautiously added in batches over 30 min to a stirred solution of 318 mg (0.96 mM) of II and 3 ml hydrazine hydrate in 20 ml EtOH under reflux. After 1 h, the solution was cooled down to room temperature, and a small amount of Raney-nickel was again added to eliminate the excess of hydrazine. After 2 h, the solution was filtered and evaporated to dryness in vacuo. The oily residue was precipitated by addition of a solution of HCl in dieth-

yloxide. The brown solid was crystallized from CH_3OH:Et_2O (50:50) giving (IV) *pd* = 277 mg, *F* = 178°C; yield, 82%.
Anal. calc. for $C_{17}H_{27}Cl_2N_3O$: C, 56.67; H, 7.55; Cl: 19.68; N, 11.66.
Found: C, 56.25; H, 7.55; Cl, 19.66; N, 11.48.
NMR δ ppm: H_4, 7.17; H_7, 7.42; H_6, 7.68; H_8, 8.3.

Compound V was obtained in the same way by Raney-nickel hydrazine hydrate reduction at room temperature for 24 h: Yield, 42%; *F* = 182°C.
Anal. found: C, 56.32; H, 7.5; Cl, 19.55; N, 11.88.
NMR δ ppm: H_4, 7.12; H_6, 7.5; H_5, 7.78; H_8, 8.29.

3-n-butyl-5-azido-1-[2-(dimethylamino) ethoxy] isoquinoline dihydrochloride, VI
and
3-n-butyl-7-azido-1-[2-(dimethylamino) ethoxy] isoquinoline dihydrochloride, VII

The two azido compounds VI and VII were synthetized by the same procedure. Due to the photosensitive nature of the final compounds, all procedures were carried out under a photographic safe light. A solution of 50 mg (0.14 mmol) of the amino derivatives IV or V in 10 ml H_2O was cooled to 5°C and adjusted to pH 1.6 with HCl. Sodium nitrite, 10.6 mg (0.15 mmol) was added and the reaction mixture was stirred for 1 h below 5°C. After this time, 10.2 mg (0.16 mmol) sodium azide were added and the solution stirred for 1 h at room temperature. After addition of 20 ml $CHCl_3$, the solution was adjusted to pH 11 with NaOH. The organic layer was separated, dried over Na_2SO_4 and evaporated to dryness in vacuo. The residual oil was dissolved in 10 ml diethyloxide and the azido compound was precipitated as brown solid by addition of 10 ml saturated Et_2O:HCl. The solid was dried in vacuo and its purity was checked by TLC and NMR spectroscopy.

VI: R_F = 0.25 in BuOH:AcOH:H_2O (4:1:1).
NMR δ ppm; H_4, 7.15; H_6, 7.35; H_7, 7.35; H_8, 7.90.

VII: R_F = 0.3 in BuOH:AcOH:H_2 (4:1:1).
NMR δ ppm; H_4, 7.02; H_6, 7.19; H_5, 7.59; H_8, 7.69.

The presence of the azide group was demonstrated by the peak at 2100 cm^{-1} in the infrared spectrum (not shown).

3-n-butyl-5-azido-1-[2-(trimethylammonium)ethoxy] isoquinoline iodide, VIII

The oily basic compound (VI), 50 mg, was dissolved in 10 ml diethyloxide, and 2 ml methyl iodide were added. The reaction mixture was stirred for 2 h at room temperature giving a precipitate which was filtered and dried in high vacuum.

VII: R_F = 0.44 in BuOH:AcOH:H_2O (9:0.5:0.5).

The presence of azido group was demonstrated by infrared spectroscopy (fig.1) and the structure determined by NMR spectroscopy. In the frequency range

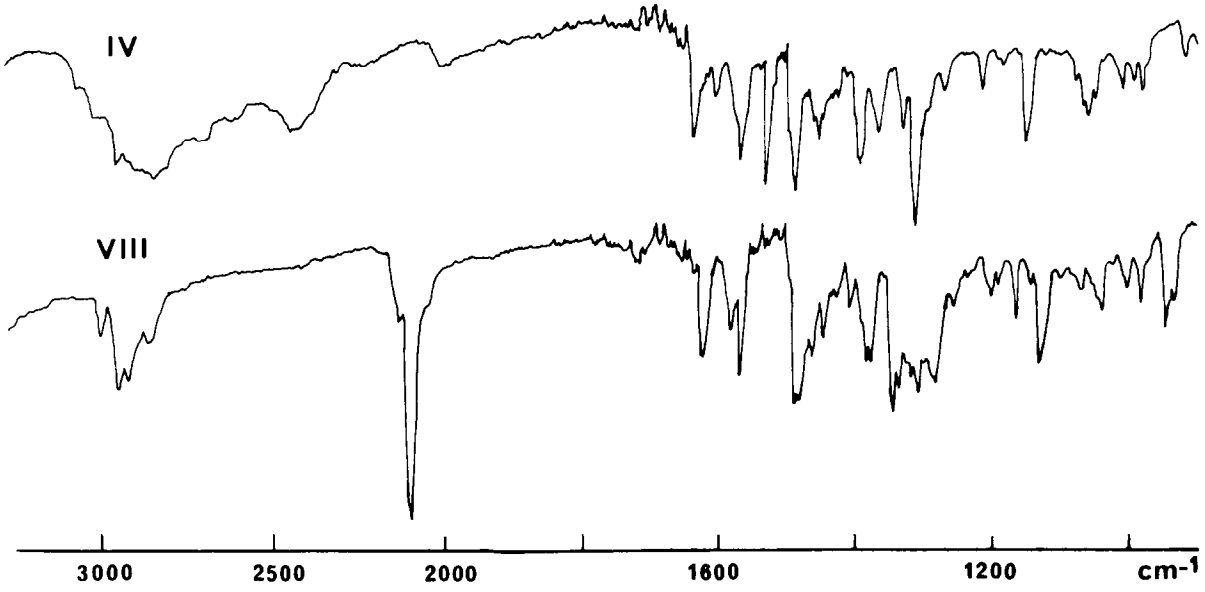


Fig.1. Infrared spectra of 5-amino dimethisoquin and 5-azido trimethisoquin, recorded in KBr (2 mg product/200 mg KBr).

of the *N*-methyl groups, only one signal at 3.25 ppm corresponding to the 9 protons of the trimethylammonium group was detected. This indicates that the pyridinic nitrogen atom in position 2 was not quaternarized because of its steric inhibition by the lateral chains located on the carbons 1 and 3.

2.2. *Electrophysiological measurements*

The pharmacological properties of the photoaffinity labelling compounds were studied in vivo on the electroplaque isolated from the Sachs organ of *E. electricus* by the method in [27]. The ligands were applied in a bath on the innervated face of the cell and the transmembrane potential recorded with an intracellular microelectrode by the method in [28].

2.3. *NMR studies*

^{1}H NMR spectra were recorded at 80 MHz on a Bruker WP 80 spectrometer operating in the CW mode and locked to the deuterium resonance of the solvent, methanol-d4. The chemical shifts (δ ppm) were measured from HMDS as internal reference.

3. Results and discussion

3.1. *NMR assigments*

Because of their relative instability, the structures of the two azido derivatives were established by NMR studies on their amino precursors IV and V. As shown in fig.2, the spectrum of compound IV shows 4 aromatic protons. The presence of only one signal (at 7.42 ppm) with 2 *ortho* coupling constants indicates that the amino group was located on the phenyl ring, in the position 5 or 8. The unambiguous characterization of the structure was performed by irradiation of the pyridinic signal at 7.17 ppm. This experiment lead to the disappearance of the *trans–trans* stereospecific long range coupling constant between H_4 and H_8 [29]. Consequently, the amino group was located on the carbon 5.

The spectrum of V (not shown here) exhibits a signal with *ortho* and *meta* coupling constants at 7.5 ppm and a quadruplet with a *meta* coupling constant at 8.29 ppm. This signal was transformed into a doublet by irradiation of the pyridinic H_4 proton, and thus corresponds to the H_8 proton. Consequently the amino group in compound V is located on the position 7.

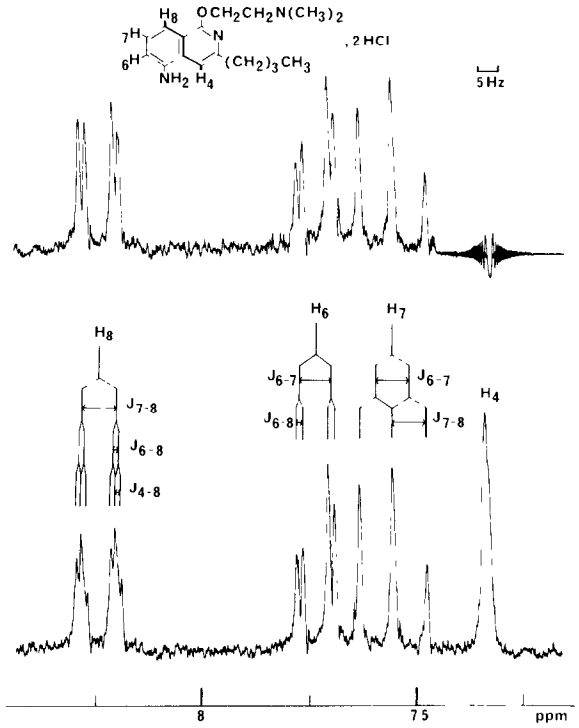


Fig.2. ^{1}H NMR spectrum of 5-amino dimethisoquin (aromatic protons), recorded at 10^{-1} M in methanol-d4.

3.2. *Pharmacological experiments with* E. electricus *electroplaque*

Figure 3a shows that bath application of 10^{-5} M 5-azido dimethisoquin to the innervated face of *E. electricus* electroplaque caused a decrease of the amplitude of the depolarisation elicited by 5×10^{-5} M carbamylcholine by 50%; after a 150 min exposure to the azide, no significant recovery of the response was noticed. In other words, the blocking effect of 5-azido dimethisoquin was irreversible.

Concentration–effect curves established for carbamylcholine after exposure of the electroplaques to 2 concentrations of 5-azido dimethisoquin showed a marked decrease of the maximal response without significant change of the apparent dissociation constant (fig.3b).

In fig.4, the irreversible blocking of the response to carbamylcholine was plotted as a function of the concentration of the various derivatives studied. With all of them, the exposure time to the innervated face of the electroplaque was 10 min. Half-maximal inhibition taking place around 10^{-5} M for both 5- and 7-azido

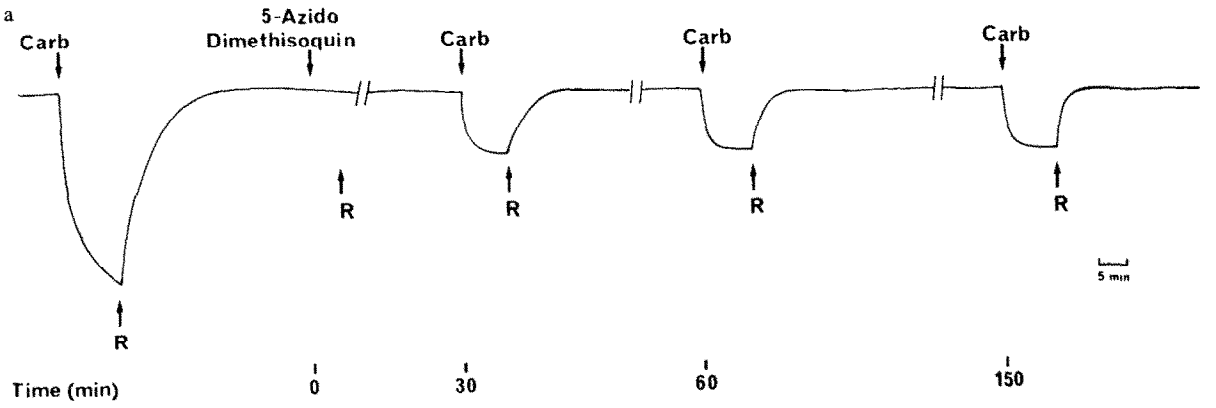
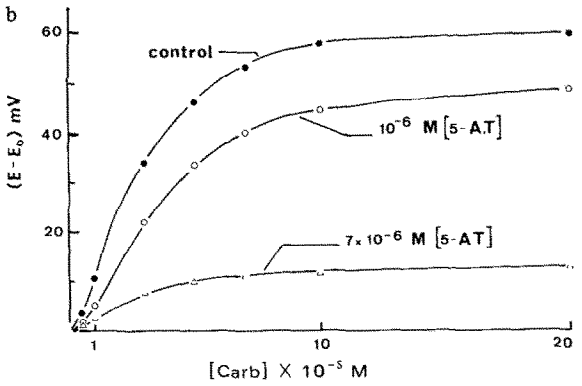


Fig.3. (a) Blocking effect of 5-azido dimethisoquin on the response to carbamylcholine of isolated electroplaque from *E. electricus*. The compound was applied in bath for 10 min at 10^{-5} M; then the cell washed with Ringer's solution. Carbamylcholine was 2.5×10^{-5} M.



(b) Effect of 5-azido trimethisoquin on the dose–response curve for carbamylcholine, 5-azido trimethisoquin was applied for 10 min. After 1 h washing with Ringer's solution, a dose–response curve was obtained by applying increasing concentrations of carbamylcholine.

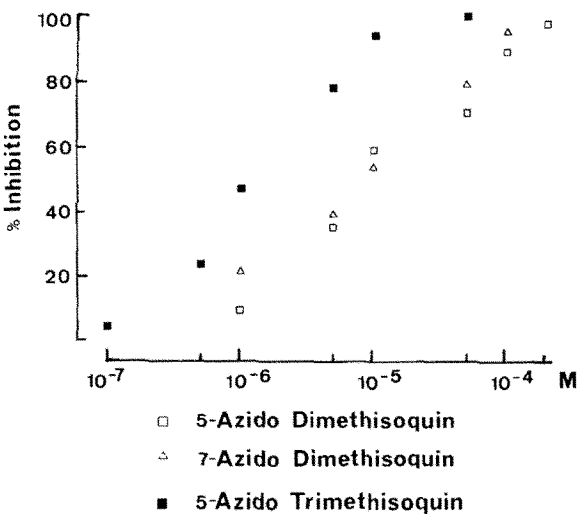


Fig.4. Blocking effect of the response to carbamylcholine (25 μM) after exposure for 10 min of the innervated face of the electroplaque to increasing concentrations of photoaffinity labelling reagents.

dimethisoquin and around 10^{-6} M for 5-azido trimethisoquin.

In conclusion, the photoaffinity labelling derivatives synthesized behave as irreversible blockers of the response of *E. electricus* electroplaque to carbamylcholine. In order to identify their membrane target(s), the binding of one of them, 5-azido trimethisoquin, was investigated after tritium labelling, with ACh receptor-rich membranes from *Torpedo marmorata* electric organ (see [25]).

Acknowledgements

The authors wish to thank Mrs S. Mougeon for her skill and initiative in performing the electrophysiological experiments, Dr M. Pesson (Lab. Roger Bellon) for supplying important amount of dimethisoquin and Mrs A. Bouju for typing the manuscript. This research was supported by grants from the Centre National de la Recherche Scientifique, l'Université René Descartes, the Délégation Générale à la Recherche Scientifique et Technique, the Collège de France, the Commissariat à l'Energie Atomique, the National Institutes of Health and the Muscular Dystrophy Association of America (from which R. O. was the recipient of a fellowship).

References

[1] Heidmann, T. and Changeux, J. P. (1978) Ann. Rev. Biochem. 47, 317–357.
[2] Lee, C. Y. (1972) Ann. Rev. Pharmacol. 12, 265–281.
[3] Changeux, J. P., Podleski, T. R. and Wofsy, L. (1967) Proc. Natl. Acad. Sci. USA 58, 2063–2070.
[4] Weiland, G., Firsman, D. and Taylor, P. (1979) Mol. Pharmacol. 15, 213–226.
[5] Karlin, A. C., Damle, V., Hamilton, S., McLaughlin, M., Valderamma, R. and Wise, D. (1979) in: Advances in Cytopharmacology (Cecarelli, B. and Clementi, F. eds) pp. 183–189, Raven Press, New York.
[6] Silman, I. and Karlin, A. (1969) Science 164, 1420–1421.
[7] Witzemann, V. and Raftery, M. A. (1978) Biochemistry 16, 5862–5868.
[8] Krodel, E. K., Beckman, R. A. and Cohen, J. B. (1979) Mol. Pharmacol. 15, 294–312.
[9] Daly, J. W., Karle, I., Myers, C. W., Tokuyama, T., Waters, J. A. and Witkop, B. (1971) Proc. Natl. Acad. Sci. USA 68, 1870–1875.
[10] Eldefrawi, A. T., Eldefrawi, M. E., Albuquerque, E. X., Oliveira, A. C., Mansour, N., Adler, M., Daly, J. W., Brown, G. B., Burgermeister, W. and Witkop, B. (1977) Proc. Natl. Acad. Sci.USA 74, 2172–2176.
[11] Weber, M. and Changeux, J. P. (1974) Mol. Pharmacol. 10, 15–34.
[12] Cohen, J. B., Weber, M. and Changeux, J. P. (1974) Mol. Pharmacol. 10, 904–932.
[13] Cohen, J. B. (1978) in: Molecular Specialization and Symmetry in Membrane Function (Solomon, A. K. and Karnowski, M. eds) pp. 99–128, Harvard University Press, Cambridge.
[14] Grünhagen, H. H. and Changeux, J. P. (1976) J. Mol. Biol. 106, 497–535.
[15] Schimerlik, M. and Raftery, M. A. (1976) Biochem. Biophys. Res. Commun. 73, 607–613.
[16] Waksman, G., Fournié-Zaluski, M. C., Roques, B. P., Heidmann, T., Grünhagen, H. H. and Changeux, J. P. (1976) FEBS Lett. 67, 335–342.
[17] Heidmann, T. and Changeux, J. P. (1979) Eur. J. Biochem. 94, 255–279.
[18] Heidmann, T. and Changeux, J. P. (1979) Eur. J. Biochem. 94, 281–296.
[19] Adams, P. R. (1977) J. Physiol. 268, 291–318.
[20] Neher, E. and Steinbach, J. H. (1978) J. Physiol. 277, 153–176.
[21] Katz, B. and Miledi, A. (1979) Symposium on the Ontogenesis and Functional Mechanisms of Peripheral Synapses, Paris.
[22] Blanchard, S. G. and Raftery, M. A. (1979) Proc. Natl. Acad. Sci. USA 76, 81–85.
[23] Sobel, A., Heidmann, T., Hofler, J. and Changeux, J. P. (1978) Proc. Natl. Acad. Sci. USA 75, 510–514.
[24] Neubig, R. R., Krodel, E. K., Boyd, N. D. and Cohen, J. B. (1979) Proc. Natl. Acad. Sci. USA 76, 690–694.
[25] Oswald, R., Sobel, A., Waksman, G., Roques, B. and Changeux, J. P. (1980) FEBS Lett. 111, 29–34.
[26] Fletcher, T. L. and Namkung, M. J. (1958) J. Org. Chem. 23, 680–683.
[27] Schoffeniels, F. and Nachmansohn, D. (1957) Biochim. Biophys. Acta 26, 1–15.
[28] Higman, H., Podleski, T. R. and Bartels, E. (1963) Biochim. Biophys. Acta 75, 187–193.
[29] Elvidge, J. A. and Foster, R. G. (1963) J. Chem. Soc. 590–592.