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Muscarinic Acetylcholine Receptors in Albino Rabbit Iris-Ciliary Body

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SUMMARY

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The binding of tritium-labeled N-methyl-4-piperidyl benzilate to homogenates of iris-ciliary body complex of albino rabbits was investigated. The binding exhibited all the characteristics typical of binding to the muscarinic acetylcholine receptor: it was saturable, of high affinity, and could be reduced by muscarinic non-labeled ligands whereas non-muscarinic drugs had no such effect. Hill coefficients were approximately 1 for the antagonists and less than 1 for the agonists. Equilibrium experiments indicated the existence of a single population of binding sites (0.44 pmol/iris-ciliary body). The formation of at least two types of ligand receptor complexes had to be assumed, however, to explain the kinetics of [^{3}H]-N-methyl-4-piperidyl benzilate binding. The simplest model which fits the experimental findings consists of a fast binding step followed by a slow isomerization of the receptor-ligand complex, as previously suggested by us for the muscarinic receptor in the mouse brain.

INTRODUCTION

The iris-ciliary body complex is known to possess a cholinergic innervation (1, 2), and has been shown to contain acetylcholine (3), cholinesterases (1, 4) and choline acetyltransferase (3, 5) in many species, including rabbit, cat, guinea pig, monkey and humans. The iris sphincter responds in a characteristic manner to muscarinic agonists and antagonists: agonists and cholinesterase inhibitors cause miosis, while muscarinic antagonists cause mydriasis. The miotic and mydriatic potencies of cholinergic drugs have been evaluated *in vivo* by topical administration (6, 7) and *in vitro* by measuring the responses of perfused iris strips (8).

While the significance of the muscarinic receptors in the iris-ciliary body complex is established, less is known about their biochemical nature. Studies of the muscarinic acetylcholine receptor in central (9, 10) and peripheral (11, 12) tissues, by means of labeled drugs, have met with success; and, based on our experience in characterizing muscarinic receptors in mouse brain homogenate (13, 14) we were able to identify the muscarinic receptor in the iris. The present work summarizes our studies on the interaction of muscarinic drugs with the receptors of nonpigmented iris, using [^{3}H]-4NMPB,[2] a highly potent and specific muscarinic antagonist (13).

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[2] The abbreviations used are: [^{3}H]-4NMPB, [^{3}H]-N-methyl-4-piperidyl benzilate; 4-NMPB, N-methyl-4-piperidyl benzilate; QNB, quinuclidinyl benzilate.

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MATERIALS AND METHODS

Materials. 4-NMPB was prepared and labeled with tritium (6 Ci/mmole) as described elsewhere (15). The chemical and radiochemical purity was determined by analytical thin-layer chromatography (Merck Silica 60 plates) in two solvent systems: n-butanol, acetic acid, water (4:1:1) and chloroform, acetone, diethylamine (5:4:1). [^{3}H]-4NMPB moved as a single peak, identical to the authentic unlabeled compound in these two systems. The purity was >97%.

The (+) and (−) isomers of QNB were prepared as described elsewhere (16). Other compounds were as follows: oxotremorine (Aldrich), scopolamine HBr (Plantex, Israel), atropine sulfate hydrate, carbamylcholine chloride, propranolol and (+)-tubocurarine (Sigma). All other compounds were of the best grade available.

Methods. Albino rabbits of either sex weighing 1.5–2.0 kg were killed by exposure to pure CO_2 in a closed chamber for three minutes. The irides were removed within 5 min, washed in ice cold Krebs solution, blotted dry and weighed. The removal of irides was done by the following procedure: a small pair of forceps was inserted through a frontal incision in the cornea so as to grasp the iris, and by gentle manipulation the entire iris together with the ciliary body, which is not developed in rabbit, was extracted intact. Twelve to 24 irides were pooled, scissor minced and homogenized in ice cold 0.32 M sucrose (12 irides/4.5 ml) using an Ultra-Turrax homogenizer (setting 7, 30 sec). They were then homogenized with a motor driven perspex pestle (950 rpm) in a glass homogenizer (5 strokes) placed in an ice bucket. The whole homogenate was centrifuged in a clinical centrifuge (2 min, 500 rpm) and the supernatant fluid collected. The pellet was rehomogenized as described (1.5 ml per 12 original irides) and recentrifuged. The supernatant was added to the former to yield the S fraction, which represents a homogenate of 2 irides/ml (~3.5 mg protein/ml). In some cases the whole homogenate was used to estimate the total binding; otherwise the S fraction was used.

Binding assay. One hundred to two hundred microliters of the prepared fractions were incubated at 25° in 2 ml modified Krebs solution (25 mM Tris·HCl, 118 mM NaCl, 4.69 mM KCl, 1.9 mM $CaCl_2$, 0.54 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 11.1 mM glucose), pH 7.4, containing the labeled or labeled and unlabeled ligands. After various incubation periods, ice cold Krebs solution (3 ml) was added and the contents were passed through a glass filter (GF/C, Whatman 25 mm diameter) by suction. The filters were washed thrice (3 ml cold Krebs solution). All procedures were completed within less than 10 sec and all binding experiments were performed in duplicates together with duplicate samples containing unlabeled drugs.

The filters were placed in vials containing 5 ml of scintillation liquid (330 ml triton X-100, 660 ml toluene, 5.5 g PPO [Packard] and 0.1 g POPOP [Merck]), maintained at 25° for 30 min, and the radioactivity then assayed by liquid scintillation spectrometry (Packard Prias Model PL). Each vial was counted for 10 min, and corrections for quenching were made by the quench curve based on the external standard ratio method using standard tritiated water and toluene (Packard).

Specific binding is defined as the total binding minus the binding in the presence of 5×10^{-5} M of 4-NMPB or atropine.

Protein was determined by the method of Lowry *et al.* (17) using bovine serum albumin as standard.

RESULTS

The binding of [^{3}H]-4NMPB at 25° was studied on the S fraction and the whole iris-ciliary body complex homogenate. The total binding in the former constituted about 80% of the binding in the latter, and the experiments were therefore carried out with the S fraction.

Equilibrium binding studies were performed at a range of concentration from 0.15 to 5 nM, shown in Fig. 1A for specific and non-specific binding of [^{3}H]-4NMPB. Specific ligand binding exhibited the hyperbolic shape typical for saturation phenomena (half saturation at 0.5 nM ligand). Nonspecific binding was much lower and varied linearly with ligand concentration. Replot-

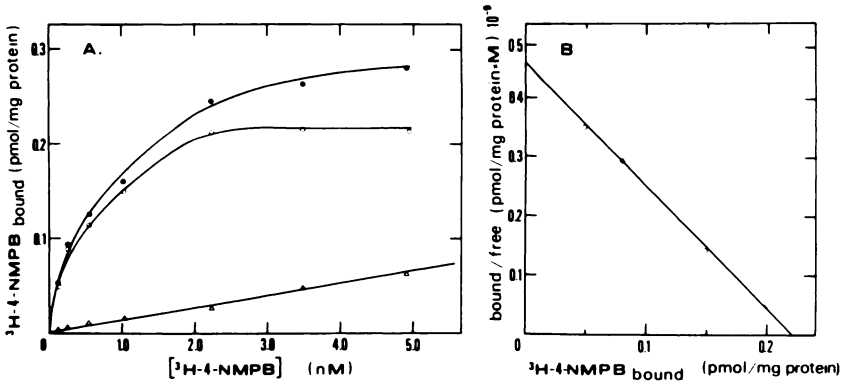

FIG. 1. *Binding of [^{3}H]-4NMPB at 25° as a function of concentration*

A. 0.2 ml S fraction samples were incubated with varying concentrations of [^{3}H]-4NMPB for 30 min in 2 ml modified Krebs solution (pH 7.4) ●——●, total binding △——△, non-specific binding (in the presence of 5×10^{-5} M 4-NMPB), ○——○, specific binding (total binding minus non-specific binding). Each experimental point represents the mean value of duplicate samples. B. Same data plotted according to Scatchard.

ting the binding data according to Scatchard (18) gave a straight line (Fig. 1B); therefore [^{3}H]-4NMPB binding reflects a single population of binding sites at the concentration range investigated. The dissociation constant determined by Scatchard analysis was 0.48 nM, and the concentration of specific [^{3}H]-4NMPB binding sites was 0.22 pmole/mg protein, or 0.44 pmole per iris.

Binding was further investigated kinetically. Figure 2 shows the time course of association of [^{3}H]-4NMPB at 2.0 nM, corrected for nonspecific binding. Half saturation was achieved in 1.5 min and maximum binding in approximately 20 min. The kinetic data were replotted according to the integrated rate equation for bimolecular reversible reaction:

$$\ln \frac{B_{eq} - B_t}{B_{eq}} = (k_1[\text{4-NMPB}] - k_{-1})t \quad (1)$$

where B_{eq} and B_t are the concentrations of 4-NMPB-receptor complexes at equilibrium and at time t, respectively. Free 4-NMPB is in great excess and therefore can be considered constant throughout the reaction. Under such pseudo first order conditions, data plotted according to this equation should yield a straight line if the reaction is bimolecular. As shown in Fig. 2 (insert) the plot deviated from linearity. Trying to fit a straight line based on least square error analysis gives an intercept (at $t = 0$) of 0.8. This deviation from the theoretical value of 1.0 is much larger than usually encountered in other experiments, indicating that the reaction is not simple bimolecular.

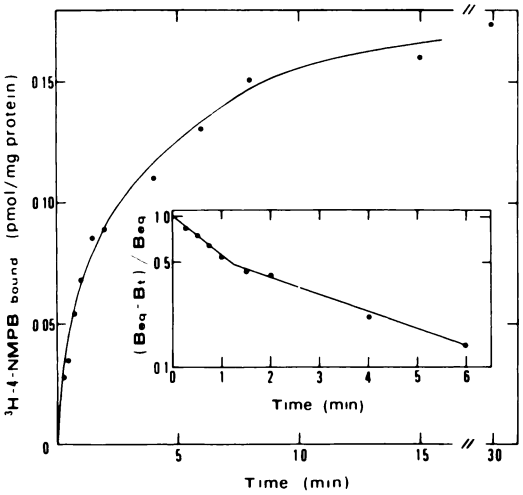

FIG. 2. *Time course of [^{3}H]-4NMPB binding at 25°*

S samples (0.2 ml) were incubated in 2 ml modified Krebs solution with [^{3}H]-4NMPB for various periods of time. Specific binding was determined as described in METHODS. Each experimental point represents the mean value of duplicate samples. Insert: same data plotted according to Eq. 1.

The dissociation of [^{3}H]-4NMPB receptor complex, measured by an isotopic dilution technique, is shown in Fig. 3. The half-time for dissociation is about 13 min.

To establish the specificity of [^{3}H]-

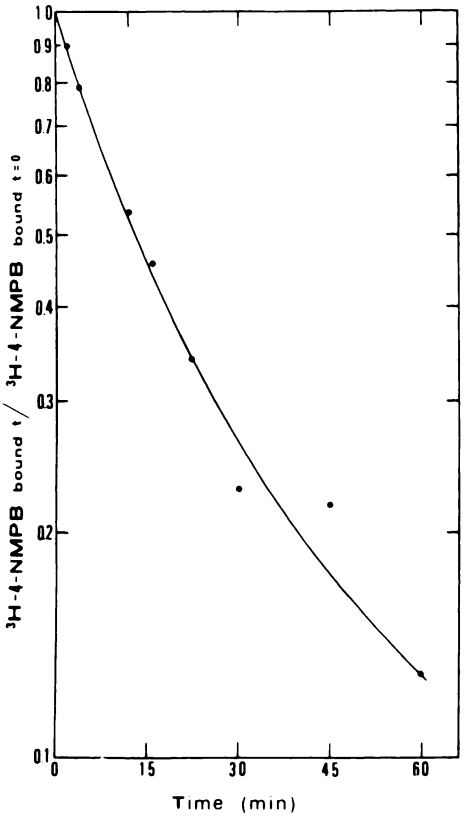

FIG. 3. *Dissociation of [^{3}H]-4NMPB at 25°*

S Samples (0.2 ml) were incubated for 30 min with 5 nM [^{3}H]-4NMPB in 2 ml modified Krebs solution. 5 × 10^{-5} M 4-NMPB was then added and the samples were filtered immediately (zero time) and at the times indicated. Specific [^{3}H]-4NMPB was determined as described in METHODS. Each point represents the mean value of two duplicate determinations ([^{3}H]-4NMPB bound)$_{t-0}$ was 0.21 pmole/mg protein.

4NMPB binding in the iris homogenate, competition experiments with unlabeled ligands were carried out (Fig. 4A). The potent muscarinic antagonists, (−) QNB, scopolamine and atropine, inhibited 50% of [^{3}H]-4NMPB binding at the nM range; the agonists oxotremorine and carbamylcholine inhibited 50% of [^{3}H]-4NMPB at 1.6 and 50 μM, respectively. Non-muscarinic drugs ((+)-tubocurarine, propranolol, haloperidol and phentolamine) at 10 μM concentration did not affect the binding. The K_d values for the muscarinic antagonists (Table 1) correlates well with their known pharmacological potencies (16, 19), as do the I_{50} values of the agonists (6). The (−) isomer of QNB is 50 times more potent than the (+) isomer, thereby establishing stereospecificity and again agreeing with the pharmacological activity of QNB isomers (16). Thus, the competition experiments demonstrated that in iris homogenate, as in the mouse brain (13), [^{3}H]-4NMPB binds specifically to muscarinic acetylcholine receptors. Hill plots of the competition curves (Fig. 4B) yielded Hill coefficients close to 1 for all antagonists tested, and less than 1 for agonists (Table 1). Such behavior has been reported previously for muscarinic receptors in several preparations (10, 11, 20, 21).

DISCUSSION

The binding of [^{3}H]-4NMPB to homogenates of albino rabbit iris-ciliary body exhibits the characteristics typical of binding to muscarinic acetylcholine receptors; that is, the binding is saturable, of high affinity (Fig. 1), and reduced by competing ligands while unaffected by non-muscarinic drugs. The relative potencies of the muscarinic drugs in competing with [^{3}H]-4NMPB binding correlates well with their pharmacological potencies (6, 7, 19). These results and previous work on 4-NMPB binding to mouse brain homogenate (13) provide strong evidence that 4-NMPB binds specifically to muscarinic acetylcholine receptors.

The concentration of receptors was found to be 0.44 pmole/iris or 0.22 pmole/mg S fraction protein. This number of receptor sites is relatively low in comparison to 2.0 pmole/mg protein in the longitudinal muscle of guinea pig ileum (12), 0.6–2.0 pmole/mg protein in different mammalian brains (9, 10, 13), and 0.4 pmole/mg protein in the chicken retina (20). On the other hand, it is close to 0.16–0.23 pmole receptor sites/mg protein in chicken heart (11), and 0.19 pmole/mg protein in the rat heart (22). Under our experimental conditions the specific binding sites concentration was 0.04–0.06 nM, or 400–600 cpm per filtered sample.

We were able to obtain reliable kinetic and equilibrium data despite the relatively small number of receptor sites because of the high affinity of 4-NMPB to these sites and the low non-specific binding in the albino rabbit iris-ciliary body. The problem

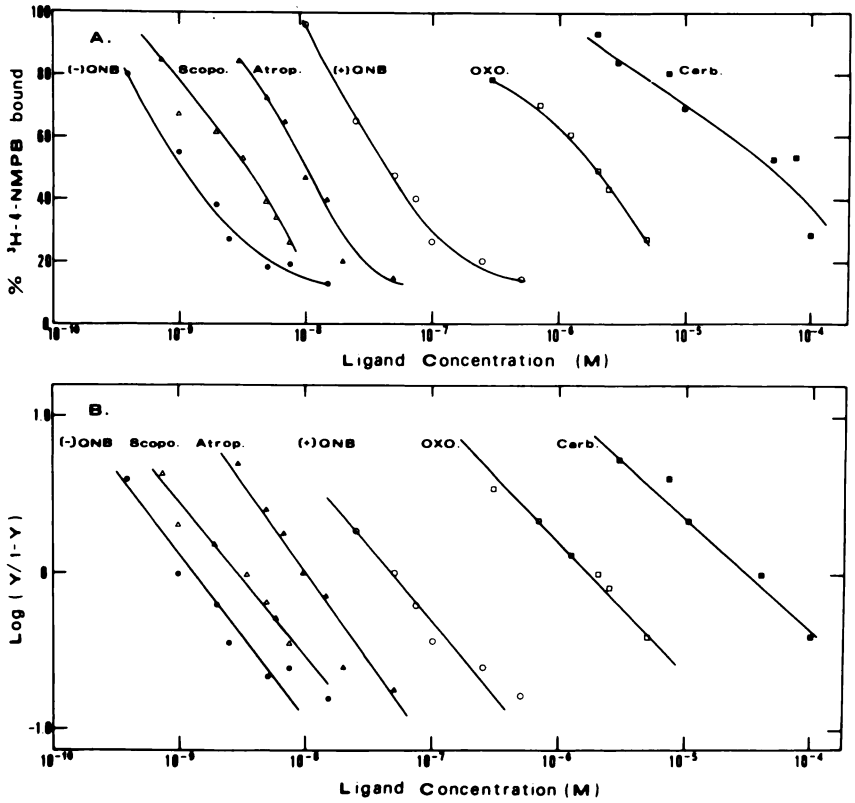

FIG. 4. *Competition binding for muscarinic drugs*

A. S samples (0.2 ml) were incubated at 25° for 30 min in 2 ml modified Krebs solution containing 1.0 nM [^{3}H]-4NMPB and the concentrations of unlabeled ligands indicated. Specific binding was determined as described in METHODS. Each point represents the average of two duplicate determination. ●——●, (−)QNB; △——△, scopolamine; ▲——▲, atropine; ○——○, (+)QNB; □——□, oxotremorine; ■——■, carbamylcholine. B. Hill plot of the same data. Y is the fractional binding of [^{3}H]-4NMPB.

of non-specific binding to pigment (23) was circumvented in this study by working with albino animals. The strong interference of non-receptor binding was observed in similar studies on non-albino animals carried out in our laboratory (23) and is in agreement with previously published information (24).

Inhibition by muscarinic agonists of antagonist binding to the receptors in smooth muscle (25), brain (10, 21), retina (20), heart (11) and iris ciliary body (Fig. 4B) preparations all gave Hill coefficients lower than 1. This behavior can be related to heterogeneity in the binding sites of agonists (10), or to negatively cooperative site-site interactions, or to desensitization (25). More detailed data should be obtained in order to distinguish between these possibilities.

The binding of labeled muscarinic antagonists to homogenates of different central and peripheral tissues show some typical characteristics. The dissociation kinetics of [^{3}H]-4NMPB, [^{3}H]-scopolamine (13) and [^{3}H]-atropine (14) in mouse brain homogenate are biphasic. Similar behavior of dissociation and association kinetics has been observed for QNB in chicken heart (11) and retina (20), and for 4-NMPB in mouse brain (26); now the same phenomenon is seen in the iris for [^{3}H]-4NMPB. Thus, the kinetics of [^{3}H]-4NMPB binding cannot be described by a simple bimolecular reaction. It is of course possible for two independent classes of sites to have similar equilibrium constants, as evident by the linear Scatchard plot, though their rate constants for the association reactions differ. This would,

TABLE 1

Binding constants and Hill coefficients (n_H) for muscarinic ligands[a]

Drug	I_{50}[b]	K_d[c]	n_H
	(M)	(M)	
[^{3}H]-4NMPB	–	$(4.8 \pm 0.4) \times 10^{-10}$	1.01 ± 0.02
(−)QNB	1×10^{-9}	$(2.9 \pm 0.5) \times 10^{-10}$	1.08 ± 0.03
(+)QNB	4.4×10^{-8}	$(1.3 \pm 0.3) \times 10^{-8}$	0.96 ± 0.05
Scopolamine	3.2×10^{-9}	$(9.2 \pm 0.2) \times 10^{-10}$	0.94 ± 0.05
Atropine	1.0×10^{-8}	$(2.9 \pm 0.5) \times 10^{-9}$	1.05 ± 0.06
Oxotremorine	1.8×10^{-6}	—	0.82 ± 0.04
Carbamylcholine	5×10^{-5}	—	0.73 ± 0.05

[a] The average values of binding constants and Hill coefficients for unlabeled drugs determined in three experiments similar to that in Fig. 4 are presented. The values for [^{3}H]-4NMPB are the mean of 4 experiments as shown in Fig. 1 (Hill plot is not shown).

[b] The concentration causing 50% reduction in binding of [^{3}H]-4NMPB (1.0 nM).

[c] Values for K_d for antagonists were calculated assuming a simple competitive interaction between [^{3}H]-4NMPB and the competing ligand as described elsewhere (13). Values for K_d for agonists depend on the mechanism responsible for the low Hill coefficients and cannot be calculated from these data.

however, require that the dissociation rate constants differ by the same ratio, a rather unlikely possibility. However, the minimal reaction sequence $R + L \rightleftarrows RL \rightleftarrows R'L$ can account for the combined data. It is tacitly assumed here that the binding of [^{3}H]-4NMPB(L) to its receptor (R) involves an isomerization step ($RL \rightleftarrows R'L$), and that this step is relatively slower than the association dissociation reactions. The data on muscarinic antagonist binding in other cases were also best fitted by this sequential model (11, 13, 14).

Evaluation of rate constants of the reactions must be based on more detailed kinetic data. However, as a preliminary approximation, the association rate constant of the reaction $R + L \rightleftarrows RL$ was estimated from the initial rate of binding (Fig. 2) to be 2.5×10^8 M^{-1} min^{-1}. The half-time for dissociation (Fig. 3) of [^{3}H]-4NMPB-receptor complex is about 13 min. These kinetic values are very similar to the values obtained from binding studies of [^{3}H]-4NMPB in mouse brain (13) and in cat iris homogenate (23), and clearly demonstrate the fast on and off rates.

Salazar and Patil have shown a prolonged mydriatic effect and retention of [^{3}H]-atropine in pigmented rabbits compared to unpigmented rabbits (24). The kinetics of [^{3}H]-4NMPB-receptor interaction in both strains is similar. We found high non-receptor binding of [^{3}H]-4NMPB to homogenates of pigmented irides (23), but not of unpigmented irides. These findings support the conclusion that muscarinic drugs are retained by the melanine granules, producing a prolonged mydriatic effect in pigmented irides, as suggested previously by Salazar and Patil (24). However, in albino rabbits the mydriatic effect of atropine is measured in hours. The relatively fast rate of dissociation of 4-NMPB from the muscarinic receptors referred to above thus suggests that other factors are responsible for the prolonged mydriatic effects of muscarinic antagonists even in albinos.

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REFERENCES

1. Csillik, B. & Koelle, G. B. (1965) Histochemistry of the adrenergic and the cholinergic autonomic innervation apparatus as represented by the rat iris. *Acta Histochem.* **22,** 35–363.
2. Wolff, E. in *Anatomy of the Eye and Orbit.* (1940) H. Lewis Publ., London, 360.
3. Consolo, S., Farattini, S., Ladinsky, H. & Thoenen, H. (1972) Effects of chemical sympathectomy on the content of acetylcholine, choline and choline acetyltransferase activity in the cat spleen and iris. *J. Physiol.* **220,** 639–646.
4. Lund-Karlsen, R. & Fonnum, F. (1976) The effect of locally applied cholinesterase inhibitors and oximes on the acetylcholinesterase activity in different parts of the guinea-pig eye. *Acta Phar-*

macol. Toxicol. **38,** 299–307.

5. Mindel, J. S. & Mittag, T. W. (1976) Choline acetyltransferase in ocular tissues of rabbits, cats, cattle and man. *Invest. Ophthalmol.* **15,** 808–814.
6. Maayani, S., Treister, G. & Sokolovsky, M. (1975) Drugs affecting the cholinergic system in the intact mammalian eye. I. Evaluation of the miotic activity of acetylcholinelike drugs in the mouse eye. *Invest. Ophthalmol.* **14,** 232–235.
7. Treister, G., Maayani, S. & Sokolovsky, M. (1975) A rapid method for measuring miotic activity of drugs in the intact mouse. *Invest. Ophthalmol.* **14,** 159–163.
8. Harris, L. S., Shimmoyo, M. & Hughes, J. (1974) Dose response of cholinergic agonists on cat irides. *Arch. Ophthalmol.* **91,** 299–302.
9. Yammamura, H. I. & Snyder, S. H. (1974) Muscarinic cholinergic binding in rat brain. *Proc. Nat. Acad. Sci. USA* **71,** 1725–1729.
10. Birdsall, N. J. M. & Hulme, E. C. (1976) Biochemical studies on muscarinic acetylcholine receptors. *J. Neurochem.* **27,** 7–16.
11. Galper, J. B., Klein, W. & Catterall, W. (1977) Muscarinic acetylcholine receptors in developing chick heart. *J. Biol. Chem.* **252,** 8692–8699.
12. Yammamura, H. I. & Snyder, S. H. (1974) Muscarinic cholinergic binding in the longitudinal muscle of the guinea pig ileum with [^{3}H]-quinuclidinyl benzilate. *Mol. Pharmacol.* **10,** 861–867.
13. Kloog, Y. & Sokolovsky, M. (1978) Studies on muscarinic acetylcholine receptors from mouse brain: Characterization of the interaction with antagonists. *Brain Res.* **144,** 31–48.
14. Kloog, Y. & Sokolovsky, M. (1978) Muscarinic binding to mouse brain receptor sites. *Biochem. Biophys. Res. Comm.* **81,** 710–717.
15. Kalir, A., Maayani, S., Rehavi, M., Elkavets, R., Pri-Bar, I., Buchman, O. & Sokolovsky, M. (1978) Structure activity relationship of some phencyclidine derivatives: *in vivo* studies in mice. *Eur. J. Med. Chem.* **13,** 17–25.
16. Rehavi, M., Maayani, S. & Sokolovsky, M. (1977) Enzymatic resolution and cholinergic properties of ±3-Quinuclidinol derivatives. *Life Sci.* **21,** 1293–1302.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) Protein measurements with the folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.
18. Scatchard, G. (1949) The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51,** 660–672.
19. Rehavi, M., Yaavetz, B., Kloog, Y., Maayani, S. & Sokolovsky, M. (1978) In vivo and in vitro studies on the antimuscarinic activity of some amino esters of benzilic acid. *Biochem. Pharmacol.* **27,** 1117–1124.
20. Sugiama, H., Daniels, M. P. & Nirenberg, M. (1977) Muscarinic acetylcholine receptors of the developing retina. *Proc. Natl. Acad. Sci. USA* **74,** 5524–5528.
21. Massoulie, J., Godwin, S., Carson, S. & Kato, G. (1977) Biochemical studies on muscarinic receptors. *Biomed.* **27,** 250–259.
22. Cavey, D., Vincent, J. P. & Lazdunsky, M. (1977) The muscarinic receptor of heart cell membranes. *FEBS Lett.* **84,** 110–114.
23. Kloog, Y., Sachs, D. I., Korczyn, A. D., Heron, D. S. & Sokolovsky, M. Muscarinic acetylcholine receptors in cat iris. *Biochem. Pharmacol.* (in press).
24. Salazar, M. & Patil, P. N. (1976) An explanation for the long duration of mydriatic effect of atropine in eye. *Invest. Ophthalmol.* **15,** 671–673.
25. Young, J. M. (1974) Desensitization and agonist binding to cholinergic receptors in intestinal smooth muscle. *FEBS Lett.* **46,** 354–356.
26. Kloog, Y., Egozi, Y. & Sokolovsky, M. (1979) Characterization of muscarinic acetylcholine receptors from mouse brain: Evidence for regional heterogeneity and isomerization. *Mol. Pharmacol.* **15,** 545–558.