Pharmacology Biochemistry and Behavior, Vol. 2, pp. 271–275. ANKHO International Inc., 1974. Printed in the U.S.A.

BRIEF COMMUNICATION

Puromycin as an Inhibitor of Rat Brain Acetylcholinesterase[1]

D. E. MOSS[2], D. R. MOSS AND D. FAHRNEY

Departments of Biochemistry and Psychology, Colorado State University, Fort Collins, Colorado 80521

(Received 26 November 1973)

MOSS, D. E., D. R. MOSS AND D. FAHRNEY. *Puromycin as an inhibitor of rat brain acetylcholinesterase.* PHARMAC. BIOCHEM. BEHAV. 2(2) 271–275, 1974. – Puromycin is an effective mixed inhibitor of rat brain acetylcholinesterase at concentrations below those used in memory studies. This kinetic analysis indicates that the concentration of puromycin which gives 50% inhibition of rat brain acetylcholinesterase at 50 μM substrate is approximately 0.5 mM. The results suggest that puromycin binds at two classes of sites on the enzyme, one of which appears to be an allosteric site. Since the allosteric site of acetylcholinesterase has properties similar to the acetylcholine receptor and appears to bind most ligands that bind to the acetylcholine receptor, this evidence supports the idea that puromycin may also block the effect of acetylcholine at the synaptic membrane. Puromycin may interfere with the recall of memory by causing direct interference with cholinergic mechanisms.

Puromycin Acetylcholinesterase Memory

THE DISCOVERY that intracranial injections of puromycin given after training could impair the retention of learned responses in mice [13] has stimulated a great amount of research. It was originally assumed that the amnestic effect of puromycin resulted from inhibition of protein synthesis in the central nervous system [13]. Subsequent experiments revealed that mixing either cycloheximide or acetoxycycloheximide, two other inhibitors of protein synthesis, with puromycin would prevent memory loss [1,11]. If the mechanism of puromycin-induced amnesia involves inhibition of protein synthesis, then the presence of a second inhibitor should have increased, not decreased, the amnestic effect. More recently, memories assumed to have been destroyed by puromycin have been reinstated by subsequent injections of a wide variety of substances into the same intracranial sites [9, 10, 12]. Therefore, the hypothesis that injections of this drug after training inhibit protein synthesis in such a way as to impair memory has become untenable.

Despite the plethora of reports on the amnestic effect of puromycin, its mechanism of action is uncertain. Although it has been suggested that this drug may interfere with memory by producing abnormal electrical activity in the brain [5] or by blocking adrenergic neurotransmission [21], there is increasing evidence that puromycin may interfere with cholinergic mechanisms as well. Wulff [24] has recently observed that puromycin blocks the postjunctional response to acetylcholine in the frog sartorius muscle preparation while Moss, Moss, and Fahrney [18] have demonstrated that puromycin is a pachycurare-like inhibitor of bovine red cell acetylcholinesterase. In this paper we present studies on the inhibition of rat brain acetylcholinesterase by puromycin. In view of the experiments which suggest that cholinergic functions are necessary for the recall of learned responses [7] and the key role acetylcholinesterase plays in neuronal function [19], our findings support the contention that puromycin may block memory recall by disruption of cholinergic mechanisms.

MATERIALS AND METHODS

Enzyme

The rat brain acetylcholinesterase (AChE) was obtained as a lipoprotein complex by Triton X-100 extraction according to the procedure of Ho and Ellman [14] except the final centrifugation was 48000 × g for one hour. The supernatant was diluted 1:50 in 0.1 M phosphate (Na) buffer, pH 7.0, containing 0.05% Triton X-100. The physical chemical characterization of this enzyme preparation has been reported [6]. The enzyme was studied in the

[1] This work was supported in part by the U. S. Public Health Service Research Grant GM 17848.

[2] Reprint requests: D. E. Moss, Department of Psychology, University of California, San Diego, P. O. Box 109, La Jolla, California 92037.

solubilized form because this preparation resulted in more reliable kinetic results than brain homogenates. Rat brain AChE solubilized by the above procedure has kinetic properties that are remarkably similar to the membrane-bound bovine red cell AChE we studied earlier [18]. The activity of butyrylcholinesterase in the whole rat brain with acetylthiocholine as substrate is negligible [8].

Assay of Acetylcholinesterase

The activity of AChE was assayed by the procedure of Ellman *et al.* [8] at pH 7.0, 25°. All assays were run in triplicate using a Beckman Kintrac VII recording spectrophotometer. The change in absorbance was linear for several minutes for a recorder range of 0.1 absorbance units. The 3.0 ml assay medium contained 2.6 ml of 0.1 M phosphate (Na) buffer, pH 7.0, containing 0.05% Triton X-100; 0.1 ml enzyme solution; 0.1 ml of 0.01 M 5′, 5′ dithio-bis-(2–nitrobenzoic acid); and 0.1 ml inhibitor solution. The cuvettes were allowed to equilibrate for 10 minutes with continuous stirring in the spectrophotometer at 25.0°. The reaction was started by the addition of 0.1 ml acetylthiocholine solution. All solutions were in 0.1 M phosphate (Na) buffer, pH 7.0, except that substrate solutions were prepared in distilled water. Enzyme solutions were stored at 4°, assayed regularly, and discarded at the first indication of a change in the value of the Michaelis constant. Enzyme solutions were not retained longer than one week.

Michaelis constants were determined by the statistical method of Wilkinson [22] using initial velocities obtained over a substrate concentration range from 25 to 250 μM. The Michaelis constant for rat brain AChE and acetylthiocholine under the above assay condition is 50 μM with a standard error of 2 percent.

Materials

Acetylthiocholine bromide and d-tubocurarine chloride pentahydrate were purchased from Sigma Chemical. Puromycin dihydrochloride was obtained from Signa Chemical and Nutritional Biochemicals. The 5′, 5′ dithio-bis-(2-nitrobenzoic acid) was from Pierce Chemical.

RESULTS

Puromycin is an effective, mixed inhibitor of AChE much like the pachycurare d-tubocurarine. The interaction of rat brain AChE with d-tubocurarine and puromycin at 50 μM substrate is illustrated by plotting v_o/v_i agains (I) where v_o is the initial velocity in the absence of inhibitor, v_i is the initial velocity in the presence of inhibitor, and (I) is the inhibitor concentration (Fig. 1). The nonlinear behavior of d–tubocurarine is characteristic of the pachycurares and these results are similar to those reported by Changeux [2] and Wombacher and Wolf [23]. AChE is known to be a multisubunit enzyme [15] and nonlinear behavior is thought to result from interactions at allosteric sites. Changeux has found pachycurares which bind only at allosteric sites; no further inhibition is observed once the allosteric sites are saturated and v_o/v_i becomes constant at higher concentrations of inhibitor. In the case of d-tubocurarine, however, v_o/v_i does not level off, and Changeux concluded that d-tubocurarine binds weakly at the catalytic sites [2]. As shown in Fig. 1, puromycin and d-tubocurarinc are qualitatively and quantitively similar inhibitors at low concentrations. Fig. 1 further suggests that puromycin inhibits AChE at more than one site like a pachycurare. These results are similar to our earlier experimental results using membrane-bound bovine red cell AChE [18].

Loftfield and Eigner [17] have discussed the use of the Hill equation for the description of enzyme inhibition involving several interacting sites. For this purpose the Hill equation is written as:

$$\log\left[\left(\frac{v_o}{v_i}\right) - 1\right] = n_H \log (I) + \text{constant}$$

where v_o is the uninhibited velocity, v_i is the inhibited velocity, and (I) is the inhibitor concentration. A plot of $\log [(v_o/v_i) - 1]$ against log (I) gives a line with slope n_H, where n_H is defined as the Hill coefficient. The Hill coefficient is a measure of the degree of cooperativity between interacting subunits of the enzyme. A Hill coefficient of 1.0 is indicative of competitive inhibition in which no interactions between binding sites exist. On the other hand, values of n_H less than 1.0 are diagnostic of negative cooperativity; that is, the existence of two or more interacting sites on the enzyme such that the binding of one inhibitor molecule makes it more difficult for the next to bind [16].

The d-tubocurarine-like inhibition of rat brain AChE by puromycin is shown by the striking similarity of the nonlinear Hill plots (Fig. 2). Although a straight line could have been fitted to the points for puromycin, the curve shown appears to reflect a change in slope at 10 μM puromycin. The n_H values for the 1–10 μM range are 0.4 for puromycin and 0.3 for d-tubocurarine. This Hill plot analysis suggests that puromycin and d-tubocurarine in this concentration range interact almost exclusively with a site topographically distinct from the catalytic site, that is, at an allosteric site. Changeux *et al.* [3] found that decamethonium bound in membrane fragments containing both AChE and acetylcholine receptors was displaced by d-tubocurarine only at concentrations above 10 μM. Their results support our inference from the Hill plots shown in Fig. 2 that, below 10 μM, d-tubocurarine and puromycin are bound almost exclusively at an allosteric site. On the other hand, inhibition at the catalytic site becomes more significant above 10 μM inhibitor as shown by n_H values which become 0.7 for puromycin and 1.0 for d-tubocurarine. Thus, the degree of cooperativity becomes less negative and approaches zero as the allosteric sites become saturated. The $I_{0.5}$ values obtained from Fig. 2 are 164 μM for d-tubocurarine and 518 μM for puromycin. Therefore, the catalytic site appears to have a higher affinity for d-tubocurarine than for puromycin.

DISCUSSION

It is well known that several antibiotics of different structural configurations and different modes of antibiotic action interfere with neuromuscular function by inhibiting prejunctional release of acetylcholine, decreasing the sensitivity of the postjunctional membrane to the depolarizing action of acetylcholine, and other effects [20]. It should not be surprising that puromycin may interfere with cholinergic neurotransmission.

Although the experiments reported here were conducted *in vitro*, the results may have implications for the interpretation of observations with puromycin *in vivo*. The injection of 180 μg of puromycin – the amount reported to

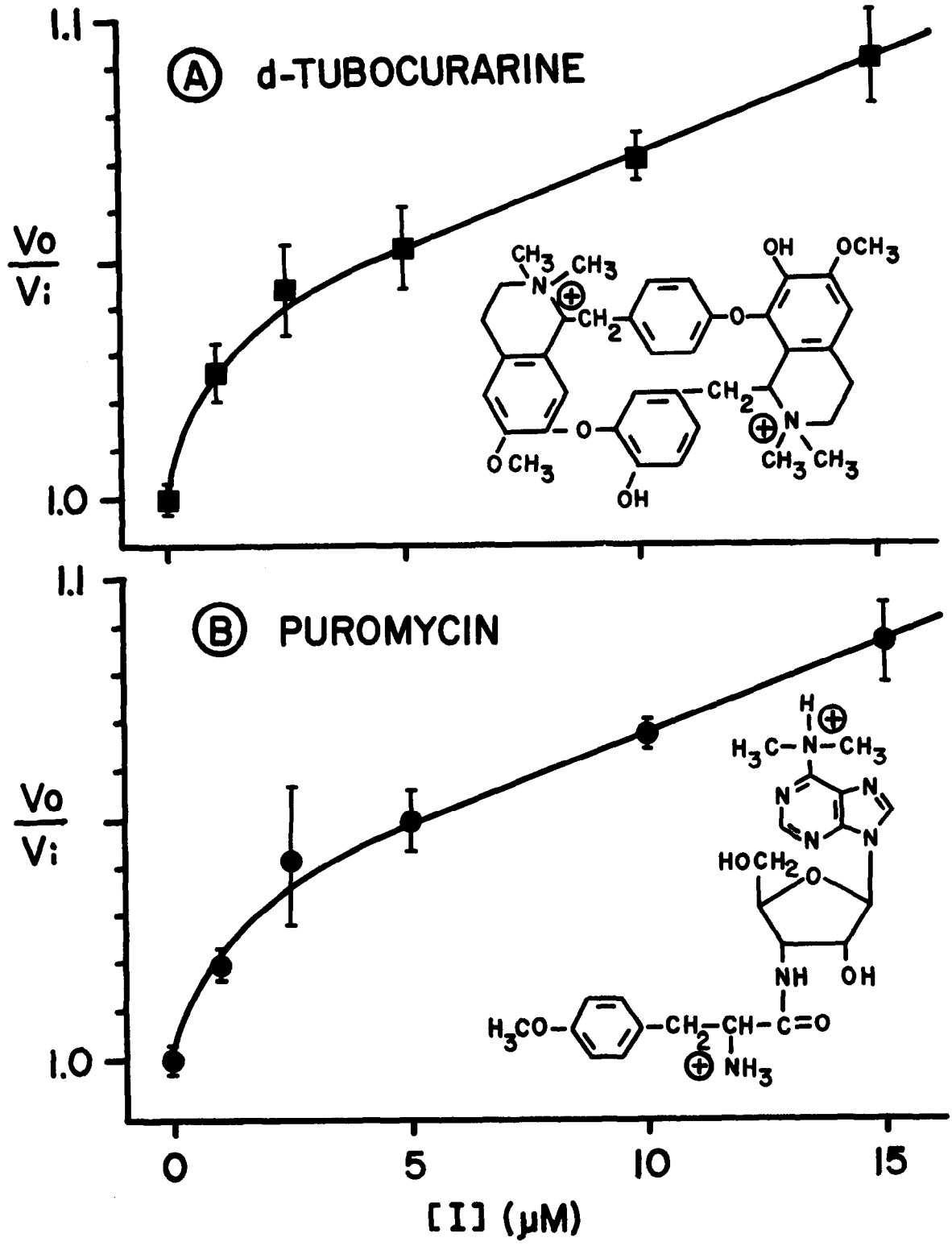

FIG. 1. Rat brain AChE interaction with d-tubocurarine and puromycin at 50 μM substrate. Initial velocity in the absence of inhibitor is v_o, initial velocity in the presence of inhibitor is v_i, and (I) is the inhibitor concentration.

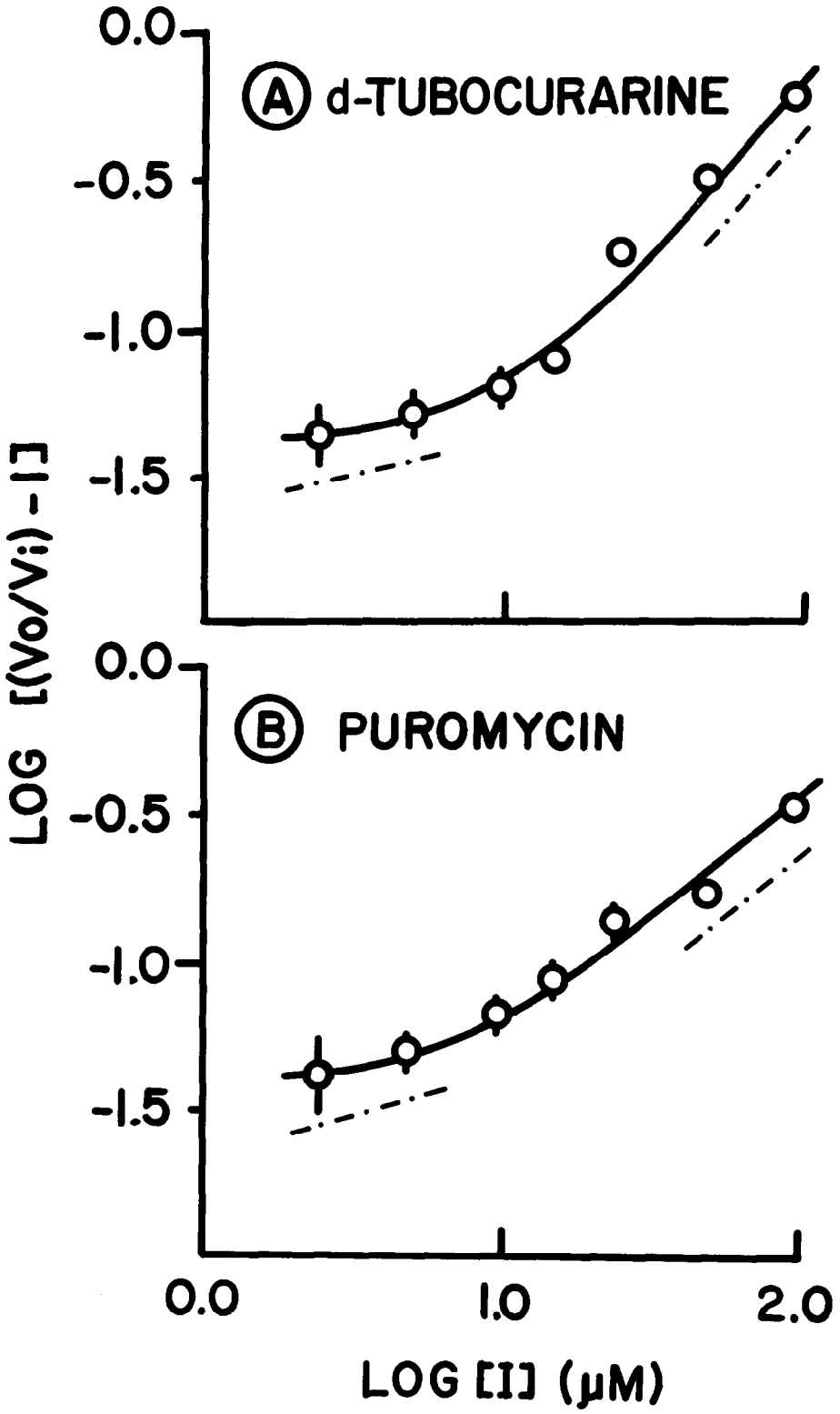

FIG. 2. Hill plot of rat brain AChE interaction with d-tubocurarine and puromycin. The Hill coefficients are as follow: 1–10 μM d-tubocurarine 0.3, 10–100 μM d-tubocurarine 1.0; 1–10 μM puromycin 0.4, 10–100 μM puromycin 0.7. The inhibitor concentration that corresponds to a value of 0.0 on the log $(v_O/v_i) - 1$ axis is the apparent $I_{0.5}$, the concentration at which $v_i = v_O/2$ at a specified substrate concentration. The $I_{0.5}$ values estimated from these plots are 518 μM and 164 μM for puromycin and d-tubocurarine respectively at 50 μM acetylthiocholine.

prevent the retention of a learned response – would result in 0.8 mM puromycin, assuming the mouse brain were a closed, homogenous 0.4 ml compartment. Because the apparent $I_{0.5}$ value for puromycin *in vitro* at 50 μM substrate was 0.5 mM, it seems reasonable to expect that puromycin has a significant effect on cholinergic transmission *in vivo*. Insofar as our earlier experiments showed that puromycin has an apparent $I_{0.5}$ of 0.13 mM with membrane-bound AChE in the bovine red cell [18], puromycin would appear to be an even more effective inhibitor of the enzyme in its natural membrane-bound form than in the solubilized form studied here. Furthermore, since the allosteric site of AChE is structurally similar to the acetylcholine receptor [4] and the allosteric site and the receptor appear to bind the same ligands [3], our data which suggest that puromycin binds at the allosteric site on AChE support Wulff's report [24] that puromycin reduces the effect of acetylcholine on synaptic membranes.

Regardless of the exact mechanism by which puromycin causes amnesia for learned responses, it is important to recognize that it may disturb cholinergic neurotransmission. It is consistent with the cholinergic hypothesis of memory as proposed by Deutsch [7] for puromycin to interfere with memory either through its effect on the acetylcholine receptor as reported by Wulff [24] or because of its effect on AChE as reported here.

REFERENCES

1. Barondes, S. H. and H. D. Cohen. Comparative effects of cycloheximide and puromycin on cerebral protein synthesis and consolidation of memory in mice. *Brain Res.* **4**: 44–51, 1967.
2. Changeux, J. P. Responses of acetylcholinesterase from *Torpedo marmorata* to salts and curarizing drugs. *Mol. Pharmac.* **2**: 369–392, 1966.
3. Changeux, J. P., J. C. Meunier and M. Huchet. Studies on the cholinergic receptor protein of *Electrophorus electricus*. *Mol. Pharmac.* **7**: 538–553, 1971.
4. Changeux, J. P., T. Podleski and J. C. Meunier. On some structural analogies between acetylcholinesterase and the macromolecular receptor of acetylcholine. *J. gen. Physiol.* **54**: 225–244, 1969.
5. Cohen, H. D. and S. H. Barondes. Puromycin effect on memory may be due to occult seizures. *Science* **157**: 333–334, 1967.
6. Crone, H. D. The dissociation of rat brain membranes bearing acetylcholinesterase by the non-ionic detergent Triton X-100 and an examination of the product. *J. Neurochem.* **18**: 489–497, 1971.
7. Deutsch, J. A. The cholinergic synapse and the site of memory. *Science* **174**: 788–794, 1971.
8. Ellman, G. L., K. Diane Courtney, V. Andres, Jr. and R. M. Featherstone. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmac* **7**: 88–95, 1961.
9. Flexner, J. B. and L. B. Flexner. Restoration of expression of memory lost after treatment with puromycin. *Proc. natn. Acad. Sci.* (U.S.A.) **57**: 1651–1654, 1967.
10. Flexner, J. B. and L. B. Flexner. Further observations on restoration of memory lost after treatment with puromycin. *Yale J. Biol. Med.* **42**: 235–240, December 1969–February 1970.
11. Flexner, L. B. and J. B. Flexner. Effect of acetoxycycloheximide and of an acetoxycycloheximide-puromycin mixture on cerebral protein synthesis and memory in mice. *Proc. natn. Acad. Sci.* (U.S.A.) **55**: 369–374, 1966.
12. Flexner, L. B. and J. B. Flexner. Intracerebral saline: effect on memory of trained mice treated with puromycin. *Science* **159**: 330–331, 1968.
13. Flexner, J. B., L. B. Flexner and E. Stellar. Memory in mice as affected by intracerebral puromycin. *Science* **141**: 57–59, 1963.
14. Ho, I. K. and G. L. Ellman. Triton solubilized acetylcholinesterase of brain. *J. Neurochem.* **16**: 1505–1513, 1969.
15. Kitz, R. J., L. M. Braswell and S. Ginsburg. On the question: is acetylcholinesterase an allosteric protein? *Mol. Pharmacol.* **6**: 108–121, 1970.
16. Koshland, D. E., Jr. The molecular basis for enzyme regulation. In: *The Enzymes*, Third Edition, Vol. I, edited by P. D. Boyer. New York: Academic Press, 1970, pp. 342–396.
17. Loftfield, R. B. and E. A. Eigner. Molecular order of participation of inhibitors (or activators) in biological systems. *Science* **164**: 305–308, 1969.
18. Moss, D. R., D. E. Moss and D. Fahrney. Puromycin as an inhibitor of acetylcholinesterase. *Biochim. Biophys. Acta*, in press.
19. Nachmansohn, D. Chemical events in conducting and synaptic membranes during electrical activity. *Proc. natn. Acad. Sci.* (U.S.A.) **68**: 3170–3174, 1971.
20. Pittinger, C. and R. Adamson. Antiobiotic blockade of neuromuscular function. *Ann. Rev. Pharmac.* **12**: 169–184, 1972.
21. Roberts, R. B., J. B. Flexner and L. B. Flexner. Some evidence for the involvement of adrenergic sites in the memory trace. *Proc. natn. Acad. Sci.* (U.S.A.) **66**: 310–313, 1970.
22. Wilkinson, G. N. Statistical estimations in enzyme kinetics. *Biochem. J.* **80**: 324–332, 1961.
23. Wombacher, H. and H. U. Wolf. Regulation of membrane-bound acetylcholinesterase activity by bisquaternary nitrogen compounds. *Mol. Pharmac.* **7**: 554–556, 1971.
24. Wulff, V. J. The effect of puromycin on neuromuscular transmission. *Pharmacol. Biochem. Behav.* **1**: 177–182, 1973.