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ACTION OF SULFHYDRYL REAGENTS ON CHOLINERGIC MECHANISMS IN SYNAPTOSOMES

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Abstract—The mechanism by which certain sulfhydryl blocking reagents affect the release of radioactive acetylcholine ([^{3}H]ACh) from synaptosomal preparations of rat cerebral cortex was studied. *N*-Ethylmaleimide (NEM) or *p*-hydroxymercuribenzene sulfonate (pHMBS), at a concentration of 0.5 mM, produced a marked release of [^{3}H]choline and [^{3}H]ACh. The release induced by these reagents was independent of $[Ca^{2+}]_0$ and was observable within 30 sec of incubation. Pretreatment for 2 min with pHMBS inhibited high affinity uptake of choline. Using a fluorescence probe, these blocking reagents were shown to cause a rapid increase of synaptosomal fluorescence, indicating that these reagents had depolarized the preparation. Although the incorporation of [^{3}H]NEM into the synaptosomal cytoplasm and particulate fractions was very rapid, an impermeant-NEM derivative (glutathione-maleimide) also release [^{3}H]ACh from the P_2 fraction, suggesting that the release of ACh by sulfhydryl blocking reagents is at least partly due to a membrane effect.

It has been shown that sulfhydryl blocking reagents are capable of increasing the release of acetylcholine(ACh) both from rat brain cortices and from Auerbach's plexus in the guinea pig ileum [1–3]. We have found recently that Juglone (5-hydroxynaphthoquinone) affected cholinergic mechanisms in synaptosomes [4] and suspected that its action was mediated by a postulated interaction of quinones with sulfhydryl groups. This observation, coupled with our interest in the effects of a variety of agents that affect release mechanisms [5–9], prompted the present study using well-characterized sulfhydryl reagents.

EXPERIMENTAL PROCEDURES

Materials. *N*-ethylmaleimide (NEM) and *p*-hydroxymercuribenzene sulfonate (pHMBS) were obtained from the Sigma Chemical Co., St. Louis, MO. *N*-[Ethyl-2-^{3}H]ethylmaleimide and [methyl-^{3}H]choline were purchased from the New England Nuclear Corp., Boston, MA. 3,3′-Dipentyl-2,2′-oxacarbocyanine was generously provided by Dr. A. S. Waggoner. Glutathione-maleimide was a gift from Dr. R. E. Abbott.

Preparation of synaptosomes. The cerebral cortex of male rats about 35 days old was used in all experiments. The crude synaptosomal fraction (P_2) was prepared as described previously [6]. Purified synaptosomes were prepared by a discontinuous Ficoll gradient, according to the method of Cotman [10]. Either the P_2 fraction or purified synaptosomes were suspended in Krebs–Ringer bicarbonate buffer (KR). The KR contained 138 mM NaCl, 5.6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 11 mM $NaHCO_3$, 1 mM NaH_2PO_4 and 10 mM glucose, and was gassed with 95% O_2–5% CO_2. The KR was adjusted to pH 7.4 by the addition of Tris base before use.

Release of ACh. The preloading of the P_2 fraction with [^{3}H]ACh was performed by a preincubation with 9.4 μM choline, containing 10 μCi/ml of [methyl-^{3}H]choline (sp. act. 10.1 Ci/m-mole). After preincubation with [^{3}H]choline for 30 min at 37°, the P_2 was pelleted by a centrifugation at 10,000 *g* for 10 min and was washed once with KR containing 0.2 mM eserine (P_2 from 1 *g* cortex/3 ml KR). Aliquots were incubated at 37° for various time intervals with or without sulfhydryl blocking reagents. The reaction was stopped by the addition of 2 mM cysteine and cooling in ice. After centrifugation, the [^{3}H]ACh in the supernatant fluid was separated from [^{3}H]choline by high-voltage paper electrophoresis [11]. The radioactivity was monitored by a liquid scintillation counter using Formula 963 (New England Nuclear, Boston, MA) as a mixture. Protein was measured by the method of Lowry *et al.* [12].

High affinity uptake of choline. The P_2 suspension in KR was preincubated at 37° for 30 min. Sulfhydryl blocking reagents were added in the final 2 min. Reactions were stopped by the addition of 2 mM cysteine and by cooling in ice. After centrifugation at 10,000 *g* for 10 min, the pellets were washed once with KR containing 2 mM cysteine and were suspended in new KR (P_2 from 1 g cortex/3 ml). After 5 min of preincubation, the reaction was started by adding 100 μl of P_2 suspension into 0.9 ml of KR-uptake medium and was carried out for 2 min. KR-uptake medium contained 0.5 μM choline and 0.5 μCi [methyl-^{3}H]choline (80 Ci/m-mole)/tube.

Sodium-independent low affinity uptake of choline was initiated in NA^+-free KR medium (Na^+ was replaced with sucrose). The uptake was stopped by rapid filtration through a Whatman GF/C filter with four washings with 4 ml of ice-cold washing solution was initiated in Na^+-free KR medium (Na^+ was removed and the P_2 pellets on the filter were solubilized by adding 0.2% Triton X-100–0.2 N NaOH. After neutralizing, the radioactivity was determined.

Membrane potentials assayed by a fluorescence

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probe. The membrane potentials of synaptosomes were indirectly monitored using the oxacarbocyanine dye, 3,3′-dipentyl-2.2′oxacarboxyanine (di-O-C_5-3), essentially according to the method of Blaustein and Goldring [13]. A P_2 fraction suspended in KR was preincubated at 37° for 30 min. One hundred μl of the P_2 suspension (protein concn, 12–15 mg/ml) was then added to 2.9 ml KR containing 7.5 μg di-O-C_2-3 and the resulting change of fluorescence intensity was recorded at various time intervals (excitation, 475 nm; emission, 500 nm). Sulfhydryl blocking reagents were added after the addition of the P_2 fraction. All the experiments were performed at 37°.

Binding of labeled N-ethylmaleimide. Purified synaptosomes suspended in KR were preincubated at 37° for 30 min. The binding of [^{3}H]NEM to the membrane was initiated by the addition of 0.5 mM NEM containing 4 μCi/ml of [^{3}H]NEM. The reaction proceeded at 37° for various time intervals, and was terminated by the addition of 2 mM cysteine in KR. After centrifugation at 10,000 *g* for 10 min, the pellet was washed three times with 2 vol. of 2 mM cysteine-KR. Three washes were enough to remove unbound [^{3}H]NEM. To hypoosmotically shock the synaptosomes, the pellet was suspended in 9 vol. of cooled distilled water, homogenized and centrifuged at 20,000 *g* for 30 min. The resulting pellet (particulate fraction) and the supernatant fraction (synaptosomal cytoplasm) were solubilized with 0.2% Triton X–100-0.2 N NaOH. The radioactivity in each fraction was determined using a liquid scintillation spectrometer.

RESULTS

Release of [^{3}H]ACh. After the P_2 fraction, which had been preloaded with [^{3}H]choline, was preincubated for 2 min, 0.5 mM *p*-hydroxymercuribenzene sulfonate (pHMBS) or NEM was added to the incubation medium, and incubation continued for 30–60 sec (Fig. 1). Under these conditions, where little spontaneous release of [^{3}H]ACh within 60 sec of incubation was observed in control tubes, the addition of 0.5 mM NEM or pHMBS markedly enhanced the release of [^{3}H]ACh from the fraction. The release of the transmitter by these reagents was initiated very rapidly, reaching a plateau within 30 sec. The activity was much more pronounced with pHMBS: the amount of [^{3}H]ACh released by pHMBS corresponded to about 80 per cent of the total [^{3}H]ACh in the preparation. In addition, the sulfhydryl reagents also enhanced release of [^{3}H]choline from P_2 fractions with the same order of potency as for

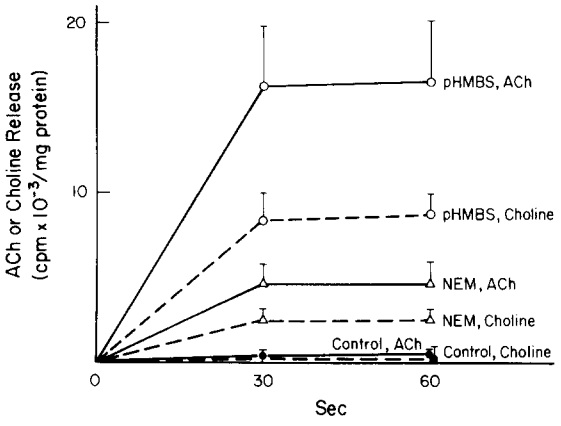

Fig. 1. Release of [^{3}H]ACh and [^{3}H]choline from a P_2 fraction by sulfhydryl reagents. After 2 min of preincubation of P_2, 0.5 mM of sulfhydryl reagents was added and incubation continued for 30–60 sec. Each point represents the mean ± S.E.M. from four separate experiments.

[^{3}H]ACh. Diamide (1 mM), an intracellular oxidizer of glutathione, also stimulated the release of [^{3}H]ACh by 50 per cent, but only after 30 min of treatment (data not shown). The release of [^{3}H]ACh by NEM or pHMBS was independent of $[Ca^{2+}]_0$ (Table 1).

The reversibility of the action of these sulfhydryl blocking reagents on [^{3}H]ACh release was tested using 2 mM cysteine. This concentration of cysteine prevented [^{3}H]ACh release by these sulfhydryl reagents if the cysteine was added to the medium simultaneously with the agents. When cysteine was added 1 min after the addition of either NEM or pHMBS, the release of [^{3}H]ACh by NEM was not affected, but by pHMBS was reduced significantly (Fig. 2).

High affinity choline uptake. Studies in many laboratories have shown that the synaptosomal preparation can accumulate choline by a high affinity Na^{2+}-dependent uptake mechanism (for review see Ref. 14). The effect of sulfhydryl blocking agents on high affinity choline uptake in synaptosomes was examined as described in Experimental Procedures. Figure 3 is a dose–response curve of the effect of pHMBS on choline uptake. pHMBS inhibited the high affinity system by approximately 75 per cent at a concentration of 0.5 mM, while showing no effect on the low affinity system (absence of Na^+). A kinetic study showed that

Table 1. Effects of calcium on the release of [^{3}H]ACh from synaptosomal preparations*

Medium	ACh release (cpm × 10^{-3}/mg protein)		
	Control	pHMBS	NEM
KR	1.0 ± 0.3	19.5 ± 4.2	5.8 ± 1.2
Ca^{2+}-free KR (+1 mM EGTA)	1.8 ± 0.5	17.3 ± 3.6	5.2 ± 2.0

* A P_2 fraction, preloaded with [^{3}H]choline, was preincubated for 1 min at 37°. After adding sulfhydryl reagents (0.5 mM), incubation was continued for 1 min. Each value represents the mean ± S.E.M. from three separate experiments.

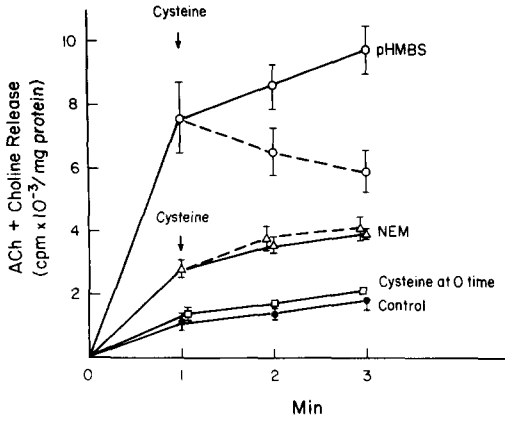

Fig. 2. Effects of cysteine on the stimulated release of [^{3}H]ACh and [^{3}H]choline by sulfhydryl reagents. The P_2 fraction was preloaded with [^{3}H]choline as described in Experimental Procedures. Cysteine (2 mM) was added either at 0 time or 1 min after sulfhydryl reagents (dotted line). Each point represents the mean ± S.E.M. from four separate experiments.

Table 2. Effects of cysteine on the inhibition by pHMBS of high affinity uptake of choline*

	Cysteine (2 mM)	Choline uptake (pmoles/mg protein)	Per cent of control
Control	+	11.7 ± 1.5	100
pHMBS (a)	−	1.2 ± 0.3	10
pHMBS (b)	+	5.0 ± 1.0	43
pHMBS (c)	+	3.6 ± 0.8	31

* Pretreatment of P_2 with pHMBS (in a final 2 min) was terminated by cooling in ice with (b, c) or without (a) 2 mM cysteine-KR. After centrifugation, pellets were washed with KR (a, b) or with 2 mM cysteine-KR (c). Each value represents the mean ± S.E.M. from three separate experiments.

pHMBS reduced the V_{max} without any change in the K_m value (data not shown).

The effect of cysteine on the inhibition of high affinity uptake of choline by pHMBS is shown in Table 2. If 2 mM cysteine was used to terminate the pretreatment of P_2 with pHMBS, the inhibition of choline uptake was reduced greatly (compare b and c to a). However, there was no further reduction of the inhibitory potency of pHMBS in a further treatment with cysteine when the latter was added to the washing solution (compare c to b).

Fluorescence probe. Recently, certain fluorescent dyes have been developed to indicate membrane potential changes in various preparations including synaptosomes from rat brain [13, 15]. The dye, 3,3′-dipentyl-2,2′-oxacarbocyanine, was used to monitor the membrane potential changes as a consequence of the addition of sulfhydryl blocking reagents. The dependence of increasing dye fluorescence on K^+ concentration has been shown to correlate well with membrane depolarization [7]. Figure 4 shows the effect of 0.5 mM pHMBSand NEM on the fluorescence intensity of synaptosomes. Only 15 sec after the addition of dye, pHMBS produced a 45 per cent increase in intensity over the control, and after 5 min that value rose to almost 95 per cent. The action of NEM was slower and less pronounced, producing a 20 per cent increase after 5 min of incubation.

Localization of action of NEM. In order to determine if the site of action of these sulfhydryl blocking reagents was on the membrane or in the synaptosomal cytoplasm, the distribution of [^{3}H]NEM in purified synaptosomes was examined (Fig. 5) No marked difference was observed in the incorporation of [^{3}H]NEM into soluble (synaptosomal cytoplasm) and synaptic membrane fractions. Figure 5 also indicates the rapidity of the binding and transport of NEM, i.e. almost 80 per cent of radioactivity was found in each fraction within only 30 sec of incubation.

Recently, new maleimide derivatives impermeant to

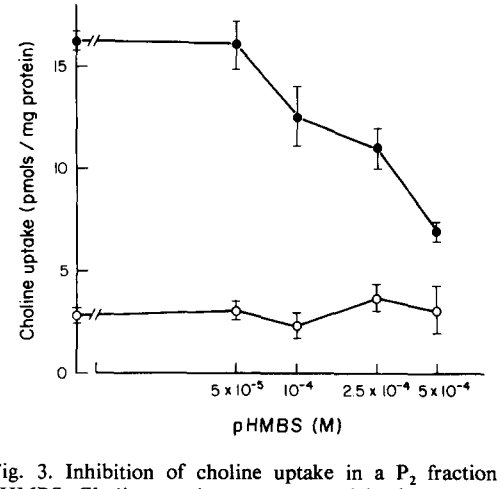

Fig. 3. Inhibition of choline uptake in a P_2 fraction by pHMBS. Choline uptake was measured in the presence of NA^+ (●——●) and the absence of Na^+ (○——○), as described in Experimental Procedures. Each point represents the mean ± S.E.M. from three separate experiments.

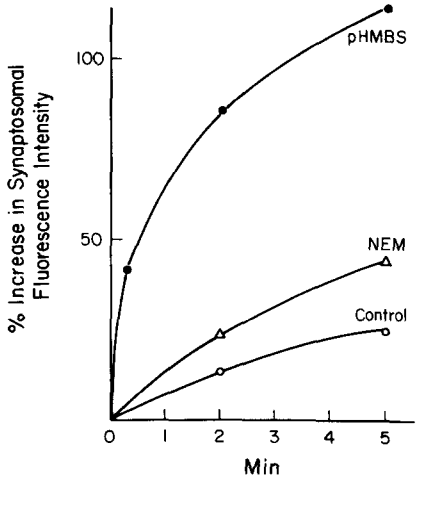

Fig. 4. Change of synaptosomal fluorescence intensity by sulfhydryl reagents. Sulfhydryl reagents were added after the addition of P_2 into the medium containing fluorescent dye, and then the time-dependent increase of synaptosomal fluorescent intensity was recorded.

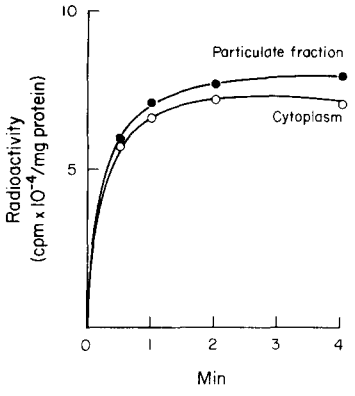

Fig. 5. Incorporation of [^{3}H]NEM into particulate and cytoplasmic fractions of purified synaptosomes. Experiments were performed as described in Experimental Procedures. Each point represents the mean value from two separate experiments.

human erythrocyte membranes have been synthesized and applied as probes for sulfhydryl groups on membranes [16, 17]. Glutathione-maleimide, an impermeant NEM, was used in an experiment on [^{3}H]choline and [^{3}H]ACh release from the P_2 fraction (Fig. 6). This agent promoted release from the fraction at a concentration of 5–15 mM. The potency of glutathione-maleimide (15 mM) was slightly less than NEM, producing a release of 60 per cent above control values.

DISCUSSION

In this study we observed that pHMBS and NEM released labeled ACh from synaptosomal preparations independently of $[Ca^{2+}]_0$ and that this release was extremely rapid; within 30 sec after the addition of

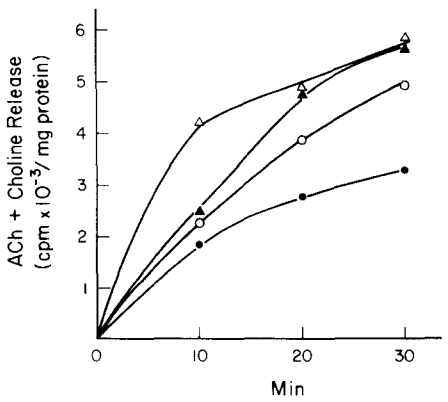

Fig. 6. Release of [^{3}H]ACh and [^{3}H]choline from a P_2 fraction by glutathione-maleimide. P_2 was preloaded with [^{3}H]choline as described in Experimental Procedures. Glutathione-maleimide was added to the P_2 suspension and incubation was carried out for various time intervals. Each point represents the mean value from three separate experiments. Key: KR (●); glutathione-maleimide: 5 mM (○), 10 mM (▲), and 15 mM (△).

pHMBS, almost 80 per cent of the [^{3}H]Ach was found in the medium. These sulfhydryl reagents also inhibited the high affinity uptake of choline. As shown in Fig. 1, the releasing activity of these agents is non-specific and non-physiological since choline is also released along with ACh. That it is high affinity choline uptake that is inhibited is indicated by the sodium dependency (Fig. 3).

Cysteine exerted a partial effect in overcoming the inhibition of choline uptake by pHMBS (Table 2). With respect to the efflux of ACh and choline induced by pHMBS, cysteine, when added 1 min after release was initiated by PHMBS, stopped the efflux. Curiously, it had no effect on release that was promoted by NEM. The explanation for this lack of effect of cysteine may be referable to the possibility that NEM had already penetrated the synaptosome and was exerting an irreversible effect on mitochondria, whereas pHMBS activity was somewhat restricted to the external synaptosomal membrane, subject to interaction with cysteine.

Since the sulfhydryl reagent-induced release of ACh could be explained on the basis that these agents depolarized the preparation, we employed the oxacarbocyanine dye technique to investigate this possibility. As noted earlier, this dye has been used to monitor membrane potential changes in a variety of cells; where it has been possible using electrophysiological recording to correlate the increase in fluorescence with depolarization, the technique has proven to be a valid indicator. However, it should be pointed out that the dye may be responding to changes other than in the membrane potential, e.g. the dye may interact with the reagent or it may be reflecting a change in pH. Bearing these caveats in mind, Fig. 4 shows that both pHMBS and NEM produce an increase in fluorescence, suggesting that the agents depolarize the preparation. In particular, the action of pMBS was very rapid, producing a 45 per cent increase in fluorescence 15 sec after it was added.

With respect to the site of action of these agents, Fig. 5 shows that NEM penetrates the organelle with extreme rapidity so that with this compound it is impossible to state where the major activity resides. For this reason we used an impermeant NEM. Using three different concentrations of glutathione maleimide, it was found that this impermeant sulfhydryl reagent promoted the efflux of ACh. In the case of pHMBS, it is not known if this agent is transported across synaptosomal membranes, although it might be assumed that sulfonic acids such as taurine have been shown to be taken up into synaptosomes by the sodium-dependent high affinity uptake system [18].

There are three findings in this work that suggest that pHMBS and NEM may have different mechanisms of action. First, the pHMBS effect was largely reversed by cysteine whereas that of NEM was not; this observation may reflect the fact that pHMBS is a reversible alkylating agent whereas the action of NEM is usually irreversible. Second, pretreatment of the synaptosomes with NEM did not inhibit the increase of fluorescence on the subsequent addition of pHMBS. Finally, effects on both release and depolarization were much more dramatic with pHMBS than with NEM.

Finally to be considered is the actual mechanism by which these sulfhydryl agents promote transmitter release. As noted in the introductory paragraph, Paton *et al.* [1] and Vizi [2, 3] have demonstrated that sulfhy-

dryl reagents promote the release of ACh from Auerbach's plexus in the guinea pig ileum and from rat brain cortices. Also, Carmody [19] has recently found that in the toad sciatic nerve-sartorius muscle preparation, both NEM and *p*-hydroxymercuribenzoate at a concentration of 0.1 mM increased ACh secretion as determined electrophysiologically. Paton *et al.* [1], whose results could not be accounted for on the basis of changes either in sodium concentration or in the membrane potential, and where calcium movements also could not be invoked in order to explain release, suggested that the Na-K ATPase was involved. Their hypothesis, based on the observations that (a) sulfhydryl reagents inhibit Na-K ATPase [20], (b) the enzyme is also inhibited by Ca^{2+} [21], and (c) the enzyme is localized in the plasma membrane [22], suggests to these authors that the Na-K ATPase activity stabilizes the nerve terminal membrane. Under normal conditions, depolarization permits the entry of Ca^{2+}, which then inhibits the Na-K ATPase, and this inhibition opens a channel to permit the egress of transmitter. Presumably, the release is terminated by the rapid removal of Ca^{2+} from its locus of action either by displacement by Mg^{2+} or by a rapid uptake into a storage site or by being pumped out of the cell. As we have shown in this study with the sulfhydryl reagents which directly inhibit Na-K ATPase, calcium is not involved. Support for this theory is supplied by experiments with ouabain, the classical inhibitor of the enzyme, where the release of ACh also was rapid and independent of calcium in the medium [2]. Moreover, Vizi [23] has shown recently in cortical slices that, by stimulating the activity of the Na-K ATPase, he can terminate the release of transmitter. Obviously, the major questions to be resolved with this speculation are: (1) how does the Na-K ATPase activity regulate channels, and (2) is the speed of the enzyme consonant with the release and termination of transmitter activity.

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REFERENCES

1. W. D. M. Paton, E. S. Vizi and M. A. Aboo Zar, *J. Physiol., Lond.* **215**, 819 (1971).
2. E. S. Vizi, *J. Physiol., London.* **226**, 95 (1972).
3. E. S. Vizi, in *Cholinergic Mechamisms* (Ed. R. G. Waser), p. 199. Raven Press, New York (1975).
4. J. R. Cooper and K. Piros, *Neurochem. Res.* **2**, 259 (1977).
5. G. P. Sgaragli, I. Sen, A. Baba, R. A. Schulz and J. R. Cooper, *Brain Res.* **134**, 113 (1977).
6. I. Sen, P. A. Grantham and J. R. Cooper, *Proc. natn. Acad. Sci. U.S.A.* **73**, 2664 (1976).
7. I. Sen and J. R. Cooper, *J. Neurochem.* **30**, 1369 (1978).
8. I. Sen, A. Baba, R. A. Schulz and J. R. Cooper, *J. Neurochem.* **31**, 969 (1978).
9. A. Baba and J. R. Cooper, *Life Sci.* **20**, 833 (1977).
10. C. W. Cotman, in *Methods in Enzymology* (Eds. S. Fleischer and L. Packer), p. 445. Academic Press, New York (1974).
11. G. P. Sgaragli and J. R. Cooper, *Biochem. Pharmac.* **23**, 911 (1974).
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
13. M. P. Blaustein and J. M. Goldring, *J. Physiol., Lond.* **247**, 589 (1975).
14. M. J. Kuhar and L. C. Murrin, *J. Neurochem.* **30**, 15 (1978).
15. C. W. Cotman, J. W. Haycock and W. F. White, *J. Physiol., London.* **254**, 475 (1976).
16. R. E. Abbot and D. Schachter, *J. biol. Chem.* **251**, 7176 (1976).
17. E. R. Batt, R. E. Abbot and D. Schachter, *J. biol. Chem.* **251**, 7184 (1976).
18. R. Schmid, W. Sieghart and M. Karobath, *J. Neurochem.* **25**, 5 (1975).
19. J. J. Carmody, *Eur. J. Pharmac.* **47**, 457 (1978).
20. J. C. Skou, *Biochem. biophys. Res. Commun.* **10**, 79 (1963).
21. J. C. Skou, *Biochim. biophys. Acta* **23**, 394 (1957).
22. J. C. Skou, *Physiol. Rev.* **45**, 596 (1965).
23. E. S. Vizi, *J. Physiol., London.* **267**, 261 (1977).