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COMPARATIVE EFFECTS OF CHLORPROMAZINE AND ITS FREE RADICAL ON MEMBRANE FUNCTIONS*

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Abstract—Chlorpromazine, chlorpromazine free radical and a sulfhydryl inhibitor, *p*-hydroxymercuribenzoate, were compared for their effects on several biochemical phenomena associated with membrane function. Chlorpromazine free radical was slightly more potent than chlorpromazine in inhibiting calcium accumulation by rat brain microsomes, but less potent in protecting synaptosomes from osmotic shock, and in inhibiting synaptosomal uptake of dopamine and norepinephrine. *p*-Hydroxymercuribenzoate failed to protect synaptosomes from osmotic shock. It was less effective than chlorpromazine and chlorpromazine free radical in inhibiting synaptosomal uptake of catecholamines. The present data indicate that biochemical effects of chlorpromazine such as the protection of synaptosomal membrane from osmotic shock and the inhibition of synaptosomal uptake of dopamine and norepinephrine are caused by chlorpromazine itself but not by chlorpromazine free radical. Although chlorpromazine free radical was a more potent inhibitor of synaptosomal calcium uptake than chlorpromazine, the differences in potency were markedly smaller than those on microsomal Na^+,K^+-ATPase previously reported from this laboratory.

The biochemical effects of chlorpromazine (CPZ) and related phenothiazine derivatives have been extensively studied in a wide variety of systems. The results indicate that these agents inhibit a number of enzymes, interfere with coenzymes, affect permeability and stability of biologic and artificial membranes, and also modify the transport of neurotransmitters at nerve endings (see reviews [1, 2]). In general, however, high concentrations of these agents are required to produce these effects and therefore it is difficult to relate such observed biochemical events with the pharmacologic action of the phenothiazine derivatives.

Several investigators have shown that free radicals of CPZ and related compounds are more potent inhibitors of enzyme activities than their respective parent compounds [3–12]. These free radicals may be produced *in vivo* from parent compounds by mechanisms such as the oxidative metabolism of the drug [13], interactions of CPZ with manganese [14] or melanin [15, 16], or the exposure of the skin or eye to light [17]. Free radicals of phenothiazine derivatives can also be generated *in vitro* by chemical or enzymatic oxidation [5, 6, 14, 18–20] or by photo-oxidation [6, 17, 21, 22], and these highly reactive species may be responsible for some of the reported biochemical effects of CPZ. Therefore, the present study was undertaken to re-examine the effects of CPZ, differentiating those of CPZ itself from those of the CPZ free radical. Since the mechanism of action of the CPZ free radical on Na^+,K^+-ATPase has been shown to involve enzyme sulfhydryl groups [9], the effect of *p*-hydroxy-mercuribenzoate (POMB), a sulfhydryl inhibitor, was also studied.

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MATERIALS AND METHODS

Tissue preparations. Male Sprague–Dawley rats weighing 200–300 g were used. Synaptosomes were prepared from whole brain homogenates as described by De Robertis *et al.* [23, 24]. Synaptosomes collected from the C-layer of a discontinuous sucrose gradient (interface between 1.0 and 1.2 M sucrose) were resuspended in 0.32 M sucrose containing 10 μM $CaCl_2$ and used for osmotic-shock studies. The crude synaptosomal fraction (fraction M of a primary subcellular fractionation [23]) was used for norepinephrine- and dopamine-uptake studies. The microsomal fraction was obtained by differential centrifugation of a 10% brain homogenate prepared in 0.32 M sucrose containing 1 mM EDTA. The supernatant obtained after centrifugation of the homogenate at 10,000 *g* for 30 min was further centrifuged at 100,000 *g* for 30 min and resuspended in 0.32 M sucrose containing no EDTA. The latter centrifugation and resuspension were repeated to remove EDTA. All preparative procedures were performed at 0–5°. Protein concentrations were assayed by the method of Lowry *et al.* [25].

CPZ free radical. In osmotic-shock and calcium-uptake studies, CPZ free radical was generated by the oxidation of CPZ in a peroxidase–hydrogen peroxide system as originally described by Cavanaugh [19] and modified by Akera and Brody [6]. In norepinephrine- and dopamine-uptake studies, the CPZ free radical in powder form, prepared as previously described from this laboratory [7], was used. These two methods produce CPZ free radicals which inhibit isolated Na^+,K^+-ATPase equi-effectively [6, 7].

Osmotic-shock studies. A 0.5-ml aliquot of a purified synaptosomal preparation (containing 2.5 mg protein in 0.5 ml) was incubated with CPZ in the presence or absence of peroxidase (4 μg/ml) and hydrogen peroxide (2 mM) at 25° for 10 min. In control tubes,

either CPZ, or the peroxidase–hydrogen peroxide was omitted. Osmotic shock was then produced by adding 3.0 ml of distilled water and incubating the mixture at 37° for 10 min. In several tubes, 0.32 M sucrose containing 10 μM $CaCl_2$ was added instead of distilled water. These tubes served as controls for osmotic shock. Synaptosomes were then collected by centrifuging the mixture at 12,000 g for 20 min. The sediment was resuspended in distilled water, and glutamic acid decarboxylase (GAD) activity was determined by the method of Lowe *et al.* [26] with precautions and checks for tissue blank and substrate concentrations as described by Baxter [27]. It has been previously shown that GAD is a soluble cytoplasmic enzyme which is released from synaptosomes during osmotic shock [28], and therefore, the loss of GAD activity from the synaptosomal pellet can be used as an estimate of the magnitude of osmotic shock [28]. All incubations in this and following experiments were performed in a dark room.

Calcium-uptake studies. Brain microsomal fractions (0.2 mg protein/ml) were incubated at 25° in the presence of 1 mM $MgCl_2$, 75 mM KCl, 5 mM Tris–ATP and 50 mM Tris–HCl buffer (pH 7.5) with or without 5 mM sodium oxalate in the presence or absence of peroxidase (4 μg/ml) and 2 mM H_2O_2 and/or various concentrations of CPZ. After a 10-min incubation, $^{45}CaCl_2$ was added to a final concentration of 10 μM. After an additional 10-min incubation, a 1.0-ml aliquot was filtered through a nitrocellulose filter (pore size, 0.8 μm; Millipore Corp. Bedford, Mass.) and rinsed with ice-cold Tris–HCl buffer. The filters were dissolved with 1.0 ml ethyleneglycol monomethyl ether and counted for bound ^{45}Ca using a liquid scintillation system. To the dissolved filter, 10 ml of a solution containing 1.67 mg *p*-bis-[2-(5-phenyl-oxazolyl)]-benzene (POPOP), 40 mg 2,5-diphenyloxazole (PPO), 2.5 ml ethyleneglycol monomethyl ether and 7.5 ml toluene was added. Counting efficiency was monitored with the external standard-channel ratio method calibrated with internal standards. A small aliquot of incubation mixture containing $^{45}CaCl$ was also counted for its radio-activity, and using this value, the radioactivity of samples was converted to pmoles of calcium.

Norepinephrine- and dopamine-uptake studies. Crude synaptosomal preparations (1.3 mg protein in a total volume of 1.0 ml) were incubated with various concentrations of drugs at 37° for 10 min in a medium containing 140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.8 mM $CaCl_2$, 1 mM sodium phosphate buffer (pH 7.4), 10 mM glucose and 20 mM Tris–HCl buffer (pH 7.4). [^{3}H]*d,l*-norepinephrine (sp. act. 4.2 Ci/m-mole) or [^{3}H]dopamine (sp. act. 10 Ci/m-mole) was then added to a final concentration of 0.3 μM and the mixture was incubated for an additional 5-min period either at 37° or 2°. The uptake of radioactive compounds was terminated by adding 9 ml of ice-cold 0.32 M sucrose and immediately centrifuging the mixture at 12,000 g for 10 min. The synaptosomal pellet was dissolved in 0.5 ml of 0.2 N KOH and then diluted to 2.0 ml with distilled water. A 1.0-ml aliquot was pipetted into a counting vial containing 10 ml of PCS solution (Amersham/Searle Corp., Arlington Heights, Ill.). The estimation of radioactivity and calculations were performed as described above. The temperature-dependent portion of catecholamine uptake, i.e. the difference between the amount of labeled compound accumulated at 37° (approximately 4000 and 7000 cpm for norepinephrine and dopamine, respectively, in control preparations) and 2° (approximately 650 and 1000 cpm for norepinephrine and dopamine, respectively, in control preparations), was calculated and the drug effect was expressed as per cent inhibition of this fraction.

Miscellaneous. $^{45}CaCl_2$, [^{3}H]*d,l*-norepinephrine and [^{3}H]dopamine were purchased from New England Nuclear, Boston, Mass. POMB, ATP and peroxidase (type I) were purchased from Sigma Chemical Co., St. Louis, Mo. Chlorpromazine and chlorpromazine sulfoxide were kindly supplied by Dr. Harry Green of Smith, Kline & French Laboratories. Other chemicals were of reagent grade. The data were analyzed for statistical significance using Student's *t*-test. The criterion for significance was a P-value of less than 0.05.

RESULTS

Osmotic-shock studies. Electron microscopy indicated that synaptosomal preparations which were incubated at 25° for 10 min, added to 0.32 M sucrose containing 10 μM $CaCl_2$, incubated at 37° for 10 min, and finally centrifuged at 12,000 g for 20 min at 0°, had apparently intact limiting membranes. A few mitochondria and reticular structures were generally observed within an area enclosed by such a membrane. After osmotic shock, similar sediments showed a picture consisting of empty membraneous structures, isolated mitochondria, and fragments of endoplasmic reticulum similar to those previously reported by De Robertis *et al.* [23, 24]. The amount of sediment was significantly smaller in preparations which were subjected to osmotic shock than in control preparations. Preliminary experiments indicated that the final pellet should be homogenized with distilled water before the GAD assay in order to obtain maximal GAD activity. Homogenization with water results in a complete rupture of synaptosomal membranes and thus GAD becomes fully accesssible to substrate and cofactors. Control GAD activity i.e. the activity of preparations which were not subjected to osmotic shock prior to the final centrifugation, but resuspended in water after the centrifugation, was 0.333 $\pm$ 0.008 μmole/mg of protein (mean $\pm$ standard error of five experiments). Osmotic shock prior to the final centrifugation produced a 38.4 $\pm$ 5.3 per cent reduction in GAD activity of water-resuspended pellets under the present experimental condition. It has been shown that this reduction of GAD activity is due to the release of GAD into the supernatant by osmotic shock [28]. CPZ at high concentrations reduced the osmotic-shock-induced loss of GAD activity and hence apparently protected the synaptosomal membrane (Fig. 1). Preincubation of synaptosomes in the presence of peroxidase and hydrogen peroxide had no significant effect on GAD activity, which was assayed after centrifugation and resuspension. This would indicate that peroxidase and hydrogen peroxide do not interfere with the GAD assay nor do they influence the osmotic shock of synaptosomes. Incubation of CPZ and synaptosomes in the presence

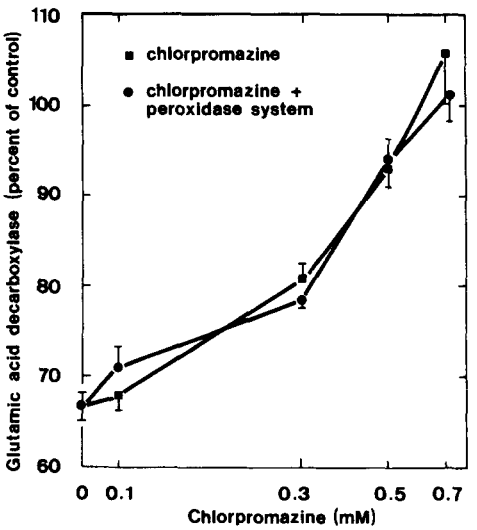

Fig. 1. Protection of synaptosomes from osmotic shock by chlorpromazine (CPZ) and CPZ free radical generated by the peroxidase–hydrogen peroxide system. Purified synaptosomes (2.5 mg protein in 0.5 ml) were incubated with various concentrations of CPZ in the presence or absence of peroxidase (4 μg/ml) and hydrogen peroxide (2 mM) at 25° for 10 min. Osmotic shock was then produced by adding 3.0 ml of distilled water. Glutamic acid decaroboxylase (GAD) activity in the synaptosomal sediment was determined, and its loss was used as an estimate of the magnitude of osmotic shock. The effect of drug was expressed as per cent recovery of GAD activity in the synaptosomal sediment after the osmotic shock, 100 per cent representing complete protection. GAD activity observed in intact synaptosomes was set at 100 per cent. Each point represents the mean of four experiments. Vertical lines indicate standard errors.

of peroxidase and hydrogen peroxide, which has been shown to convert a significant portion of CPZ to CPZ free radical and ultimately to CPZ sulfoxide [6], did not enhance the effect of CPZ (Fig. 1). Spectrophotometric analyses of the incubation mixture after the initial 10-min incubation at 25° indicated the conversion of CPZ to CPZ sulfoxide (data not shown). Thus, CPZ was capable of protecting synaptosomes from osmotic shock. Conversion of CPZ to CPZ free radical by enzymatic oxidation failed to enhance the protective effects of CPZ. POMB failed to protect synaptosomes from osmotic shock at all concentrations studied. At concentrations higher than 0.1 mM, POMB appeared to enhance the osmotic-shock-induced release of GAD (data not shown).

Calcium-uptake studies. The ability of brain microsomes to actively accumulate calcium in the presence of $MgCl_2$ and ATP has been reported by numerous investigators and is postulated to play an important role in the normal function of nerve cells which require low intracellular Ca^{2+} concentrations in the resting state (for example, see [29]). Under the present experimental conditions, accumulation of ^{45}Ca by brain microsomal preparations in the absence of CPZ was 1.81 ± 0.18 and 3.60 ± 0.69 nmoles Ca/mg of protein (mean ± standard error of six experiments) in the absence and presence of 5 mM sodium oxalate respectively. The presence of peroxidase and hydrogen peroxide had no effect on microsomal calcium uptake. CPZ at high concentrations produced a marked inhibition of calcium accumulation, either in the absence or presence of oxalate (Fig. 2, panels A and B). The slight stimulation of microsomal calcium accumulation observed with low concentrations of CPZ was not statistically signifi-

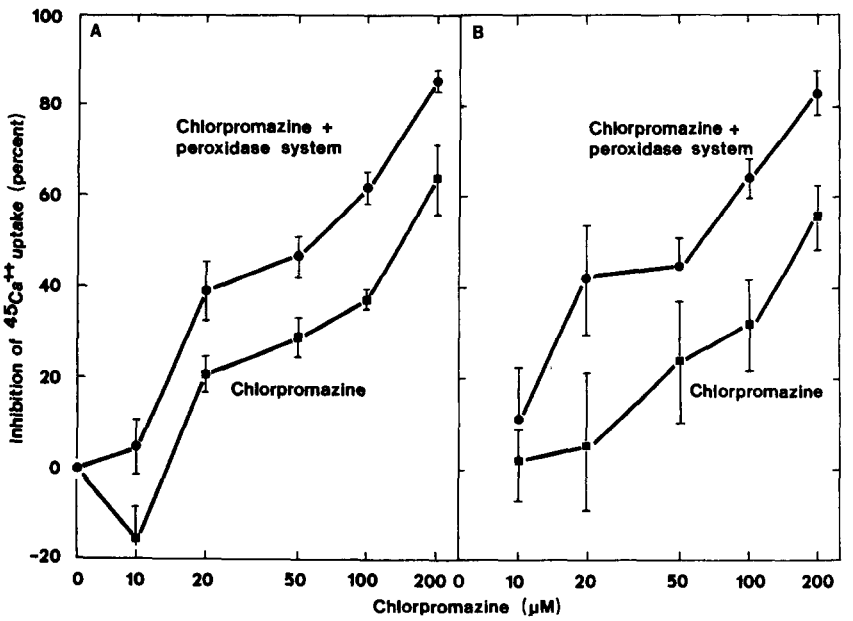

Fig. 2. Inhibition of microsomal ^{45}Ca uptake by CPZ and CPZ free radical. Brain microsomal preparations (0.2 mg protein/ml) were incubated with 1 mM $MgCl_2$, 75 mM KCl, 2.5 mM Tris–ATP and 50 mM Tris–HCl buffer (pH 7.5) for 10 min at 25° in the presence or absence of peroxidase (4 μg/ml) and hydrogen peroxide (2 mM). $^{45}CaCl_2$ (final concentration, 10 μM) was then added and incubated for 10 min at 25°. Panel A: no oxalate added to the mixture. Panel B: 5 mM sodium oxalate present. Values are expressed as per cent inhibition of ^{45}Ca uptake. Each point represents the mean of six experiments. Vertical lines indicate standard errors.

cant. Incubation of CPZ and microsomal preparations in the presence of peroxidase and hydrogen peroxide prior to the assay of calcium accumulation enhanced the inhibitory effect of CPZ. Again oxalate had no effect on the enhanced inhibition of microsomal calcium accumulation. Thus, both CPZ and CPZ free radical inhibited microsomal calcium accumulation. CPZ free radical was slightly more potent than the parent compound.

Catecholamine-uptake studies. Catecholamines have been shown to be concentrated in synaptosomes by an active transport mechanism [30, 31]. This phenomenon represents the uptake of catecholamines into nerve terminals and thus plays an important role in synaptic transmission. K_m values for the active uptake of *d,l*-norepinephrine and dopamine have been reported to be 0.57 and 0.40 μM respectively [31, 32]. Therefore, the uptake of *d,l*-norepinephrine and dopamine was assayed in concentrations of 0.3 μM of these drugs, values slightly below the K_m. The accumulation of catecholamines by synaptosomal preparations was linear with time for at least 8 min at 37°, whereas the amount of catecholamines accumulated by synaptosomal preparations did not significantly increase with time at 2°. Under the present experimental conditions, the accumulation by control synaptosomal preparations of *d,l*-norepinephrine and dopamine was approximately 10.1 and 16.2 pmoles/mg of protein, respectively, at 37° and 1.56 and 2.86 pmoles/mg of protein, respectively, at 2°. Thus, the temperature-dependent accumulation of *d,l*-norepinephrine and dopamine was 8.58 ± 0.45 and 13.4 ± 0.51 pmoles/mg of protein respectively (mean ± standard error of twenty experiments). CPZ at concentrations of 15–20 μM inhibited 50 per cent of the temperature-dependent uptake of *d,l*-norepinephrine and dopamine by synaptosomal preparations (Figs. 3 and 4). Chemically prepared CPZ free radical was slightly less effective than CPZ. POMB was also less effective than CPZ in these studies. CPZ sulfoxide was the least potent inhibitor of synaptosomal catecholamine uptake (Figs. 3 and 4).

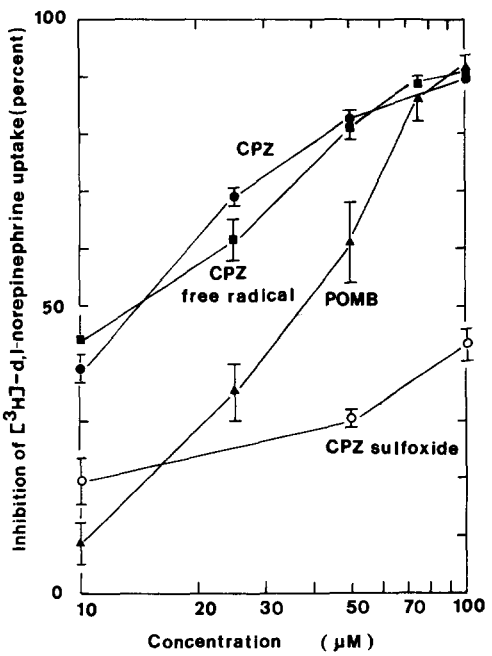

Fig. 3. Inhibition of synaptosomal *d,l*-norepinephrine uptake by CPZ, CPZ free radical, CPZ sulfoxide and POMB. Crude synaptosomes (1.3 mg in 1.0 ml) were incubated with various concentrations of drugs at 37° for 10 min. [^{3}H]*d,l*-norepinephrine was then added to a final concentration of 0.3 μM and the mixture was incubated for an additional 5-min period either at 37° or 2°. Temperature-dependent *d,l*-norepinephrine uptake, i.e. the difference in the amount of [^{3}H]*d,l*-norepinephrine accumulated at 37° and 2°, was calculated and the effect of the drug was expressed as per cent inhibition of this fraction. Each point represents the mean of five experiments. Vertical lines indicate standard errors.

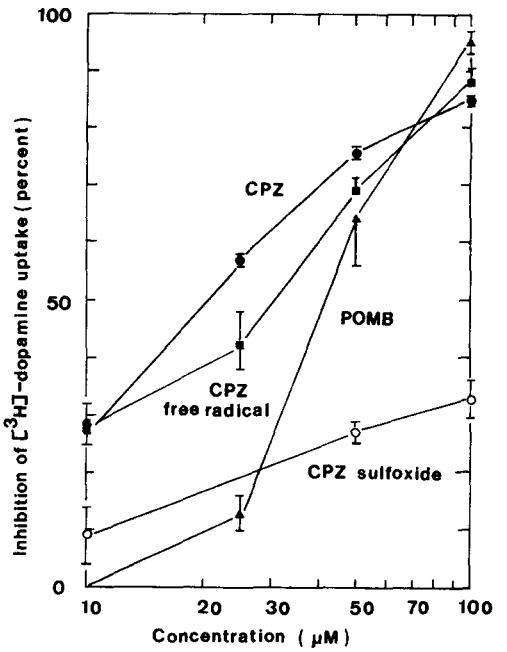

Fig. 4. Inhibition of synaptosomal dopamine uptake by CPZ, CPZ free radical, CPZ sulfoxide and POMB. See legend to Fig. 3. [^{3}H]dopamine (0.3 μM) was used in these studies.

DISCUSSION

CPZ free radical has been shown to be a more potent inhibitor of both brain microsomal Na^+,K^+-ATPase and cholinesterase activities than is the parent compound, CPZ [10]. The mechanism of action of CPZ free radical on Na^+,K^+-ATPase appears to involve essential sulfhydryl groups on the enzyme [9]. The inhibition of microsomal Na^+,K^+-ATPase by CPZ free radical is irreversible [8].

In the present study, CPZ free radicals were also slightly more potent than CPZ in inhibiting brain microsomal calcium accumulation. The inhibition of microsomal calcium accumulation by CPZ free radical may also involve sulfhydryl groups, since sulfhydryl blocking agents have been shown to decrease calcium accumulation by brain microsomes [33]. Recently, O'Callaghan and Duggan [34] reported that CPZ free radical inhibits, but CPZ itself activates, the calcium uptake by isolated microsomal fractions obtained from rabbit skeletal muscle. It is

unknown if the differences in effects of CPZ are due to the source of the microsomal fractions or other factors.

CPZ free radicals were less potent than CPZ itself in protecting synaptosomes from osmotic shock and in inhibiting synaptosomal uptake of *d,l*-norepinephine and dopamine. CPZ sulfoxide was much less effective than these compounds in the latter two systems. It should be noted that two different methods were used to prepare CPZ free radical in these studies. However, the CPZ free radical prepared by either method has been shown to be a potent inhibitor of microsomal Na^+,K^+-ATPase activity [10], and in fact ultraviolet and visible spectroscopy demonstrated that CPZ free radical prepared via the peroxidase–hydrogen peroxide system and that produced chemically are identical [10].

POMB, a sulfhydryl inhibitor, failed to protect synaptosomes from osmotic shock at concentrations lower than 0.1 mM. At concentrations higher than 0.1 mM, it caused a release of GAD from synaptosomes and, thus, failed to mimic the action of CPZ or CPZ free radical. These results would suggest that the observed protection of synaptosomes from osmotic shock is due to CPZ itself rather than to the CPZ free radical.

POMB was relatively ineffective in inhibiting synaptosomal catecholamine uptake, and CPZ free radical was less effective than CPZ. This latter result was rather unexpected since Na^+,K^+-ATPase, which is highly sensitive to CPZ free radical [10], has been implicated in catecholamine uptake [30]. Sulfhydryl groups on synaptosomes which are essential for catecholamine uptake seem to be inaccessible to the short-lived CPZ free radical. It has been shown previously that CPZ free radicals have a short life in aqueous solution at neutral pH, although the free radicals prepared using the present methods were capable of interacting with Na^+,K^+-ATPase *in vitro* [6, 7, 9, 10]. This would indicate that free radicals exist long enough to produce significant interactions with biological molecules before complete dismutation when reactive groups are readily accessible. If reactive groups are not exposed to the outer aspect of the synaptosomal membrane, however, such interactions may not occur. Whether such sulfhydryl groups are located at the inner aspect of the cytoplasmic membrane has yet to be determined.

The present study indicates that the biochemical effects of CPZ such as the protection of synaptosomal membrane from osmotic shock and the inhibition of synaptosomal uptake of catecholamines are caused by CPZ itself but not by the CPZ free radical. CPZ free radical was a more potent inhibitor of synaptosomal calcium uptake than was CPZ. However, the differences in potency were minimal compared to the effects of CPZ and CPZ free radical on microsomal Na^+,K^+-ATPase or cholinesterase activities [10]. Thus, it would appear unlikely that the observed activity of CPZ is due to CPZ free radical generated in the test tube, in contrast to Na^+,K^+-ATPase inhibition by CPZ where previously reported enzyme inhibition [35] appears to be due to CPZ free radical generated by incident light *in vitro* [6].

CPZ free radical is a relatively specific inhibitor of Na^+,K^+-ATPase. Whereas microsomal Na^+,K^+-ATPase was inhibited by 50 per cent with less than 10^{-6} M CPZ free radical [6], such a concentration of CPZ free radical failed to influence several biochemical systems which are involved in cytoplasmic membrane function.

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