Biochemical Pharmacology, Vol. 21, pp. 1403–1411. Pergamon Press, 1972. Printed in Great Britain.

EFFECTS OF CHLORPROMAZINE FREE RADICAL ON BRAIN MICROSOMAL ENZYMES*

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(*Received* 4 *February* 1971; *accepted* 5 *November* 1971)

Abstract—The inhibitory effect of chlorpromazine free radical on microsomal enzyme activities was compared using a deoxycholic acid-treated, rat brain microsomal fraction. The chlorpromazine–microsome mixture was exposed to ultraviolet light to obtain the chlorpromazine free radical-inhibited microsomal preparation. $(Na^+ + K^+)$-ATPase [Mg^{2+}-dependent, $(Na^+ + K^+)$-activated ATP phospho-hydrolase, EC 3.6.1.3] activity was the most sensitive to the inhibitory effect of chlorpromazine free radical among the enzyme activities studied. Cholinesterase (acetylcholine acyl-hydrolase, EC 3.1.1.8) activity was significantly less sensitive, and Mg^{2+}-ATPase (Mg^{2+}-dependent ATP phospho-hydrolase, EC 3.6.1.3) and NADH-cytochrome *c* reductase (NADH: cytochrome *c* oxido-reductase, EC 1.6.2.1) activities were rather insensitive to chlorpromazine free radical. The inhibition of $(Na^+ + K^+)$-ATPase and cholinesterase activities progressed even after the dissipation of chlorpromazine free radical, whereas the inhibition of less sensitive NADH-cytochrome *c* reductase activity did not progress with time. A non-enzymatic reduction of cytochrome *c* by chlorpromazine free radical was observed. We conclude that chlorpromazine free radical selectively inhibits microsomal enzymes, and the selectivity seems to depend on the difference in affinity of these enzymes for chlorpromazine free radical.

WE HAVE previously shown that chlorpromazine free radical is a potent inhibitor of brain microsomal $(Na^+ + K^+)$-ATPase activity.[1,2] This molecular species may be produced in the brain from chlorpromazine by mechanisms such as the oxidative metabolism of the drug[3] or by interaction of chlorpromazine with manganese[4] or melanin.[5,6]

In a subsequent paper,[7] we have demonstrated that the mechanism of action of chlorpromazine free radical on $(Na^+ + K^+)$-ATPase may be the inhibition of free sulfhydryl groups of the enzyme. Since free sulfhydryl groups are essential for the activity of many enzymes and the inhibition of several enzyme systems by chlorpromazine free radical has been reported,[1,2,7–10] it was of interest to test the selectivity of chlorpromazine free radical on certain enzyme systems.

In the present study, chlorpromazine free radical was generated by ultraviolet exposure, and its effects on brain microsomal enzymes were compared. The inhibition of the $(Na^+ + K^+)$-ATPase by chlorpromazine free radical is irreversible, although the inhibited enzyme can be reactivated by the treatment with NaCl or sulfhydryl reagents.[2] Thus, the enzyme concentration, or the concentration of the inhibitory sites, is a factor in determining the magnitude of the inhibition.[11] Therefore, it is meaningless to compare the effects of chlorpromazine free radical on a wide variety of purified enzymes in which the concentration of the binding sites for chlorpromazine

* This work was supported by Grant 5-RO1-MH12783-05 from the National Institute of Mental Health.

free radical may not be uniform. Therefore, only microsomal enzymes, namely NADH-cytochrome *c* reductase, $(Na^+ + K^+)$-ATPase, Mg^{2+}-ATPase and cholinesterase were selected in the present study, because these enzyme activities can be compared using the same microsomal preparation after the treatment with chlorpromazine free radical. The first two enzymes contain essential sulfhydryl groups,[12,13] whereas the reactive site for the fourth enzyme does not.[14] Mg^{2+}-ATPase activity was added for comparison with $(Na^+ + K^+)$-ATPase. Sulfhydryl groups appear to be essential for this enzyme activity[15] although some investigators found this enzyme to be insensitive to some types of sulfhydryl-inhibiting reagents.[16,17]

METHODS

Brains from male Sprague–Dawley rats, weighing 200–300 g, were homogenized in a solution containing 0·32 M sucrose, 5 mM histidine, 5 mM EDTA and 0·15 per cent sodium deoxycholic acid, and the microsomal fraction was obtained by differential centrifugation.[1] A mixture containing 50 μg of microsomal protein in 2·5 ml of 100 mM tris–HCl buffer (pH 7·5) and 0, 2, 10 and 50 μM chlorpromazine–HCl was exposed to ultraviolet light (primary emission wavelength 253·7 nm) as described previously[1] to generate chlorpromazine free radical. When ultraviolet light exposure was performed under anaerobic conditions, the mixture was placed in a Thumberg-type quartz cuvette (Precision Cells, Inc., Hicksville, N.Y.) and gassed with a nitrogen stream for 10 min prior to exposure. To insure anaerobic conditions during the exposure period, 0·8 ml of freshly prepared 2·5 per cent pyrogallol in 40% KOH was placed in the side-arm of the cuvette.[18] When the effect of chlorpromazine was studied, a similar mixture was kept in a dark place for the same period. After appropriate dilution of the above mixture, ATPase activities were estimated as described.[1] Cholinesterase activity was assayed by the method of Ellman *et al.*[19] at pH 7·8. NADH-cytochrome *c* reductase activity was assayed by the method of Lehman and Nason.[20] The amount of cytochrome *c* reduced was calculated according to Massey.[21] The measurement of absorbance for cholinesterase and NADH-cytochrome *c* reductase assays was performed with a Shimadzu Model MPS-50L dual-beam recording spectrophotometer equipped with a constant temperature attachment (American Instrument Company, Inc., Silver Springs, Md.) The results were analyzed for significance by the Student's *t*-test.

To assure the same experimental conditions, all enzyme activities were assayed at 37° after a 5-min preincubation period. When the microsomal preparation was exposed to ultraviolet light, the preincubation was started immediately after the termination of the 4-min exposure. All control reactions were linear with time during the assay period. Butyrylthiocholinesterase and cytochrome *c* oxidase activities were negligible in the present microsomal preparation.

RESULTS

Concentrations up to 10 μM chlorpromazine produced little effect on these enzyme activities without ultraviolet exposure (Fig. 1). At chlorpromazine concentrations of 50 μM, $(Na^+ + K^+)$-ATPase and cholinesterase activities were slightly inhibited, although Mg^{2+}-ATPase and cytochrome *c* reductase activities were unchanged (Fig. 1). In these experiments, exposure of the chlorpromazine–microsome mixture to light was minimal. However, it was not possible to determine whether this small

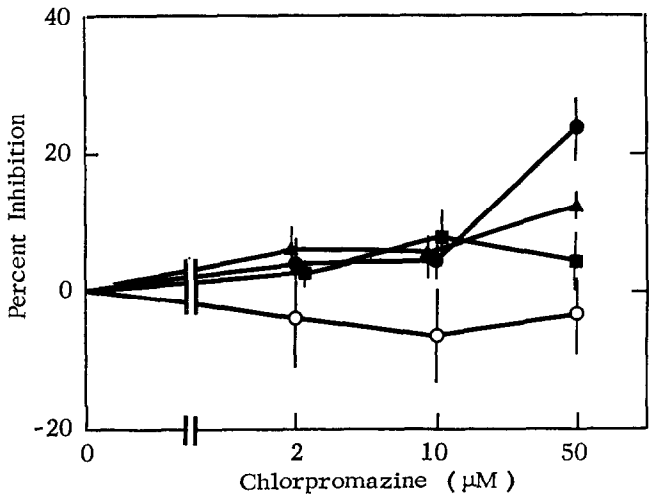

FIG. 1. Effect of chlorpromazine on microsomal enzyme activities without ultraviolet exposure. A mixture containing 50 μg of microsomal protein and various concentrations of chlorpromazine as indicated in 2·5 ml of 100 mM tris–HCl buffer (pH 7·5) was stored in a dark place for 4 min at 25°. The mixture or its aliquots were assayed for various enzyme activities at 37° after a 5-min preincubation period. The per cent inhibition of the enzyme activities was calculated against the control enzyme activities which were assayed in the absence of chlorpromazine. Mean of eight experiments. Vertical line indicates S.E.M. ●——● $(Na^+ + K^+)$-ATPase. ○——○ Mg^{2+}-ATPase. ▲——▲ Cholinesterase. ■——■ NADH-cytochrome *c* reductase.

inhibitory effect of chlorpromazine was due to the chlorpromazine free radical or to chlorpromazine itself, since exposure to light could not be avoided completely during the experiments.

The ultraviolet exposure in the absence of chlorpromazine decreased all enzyme activities by 13–20 per cent (Table 1). Figure 2 shows the inhibition of enzyme activities observed after the ultraviolet exposure of the chlorpromazine–microsome mixture. It should be noted that the drug concentration refers to the chlorpromazine added to each mixture, although the actual inhibitory species is the chlorpromazine free radical.

TABLE 1. MICROSOMAL $(Na^+ + K^+)$-ATPASE, Mg^{2+}-ATPASE, CHOLINESTERASE AND NADH-CYTOCHROME *c* REDUCTASE ACTIVITIES AND THE EFFECT OF ULTRAVIOLET EXPOSURE*

	Control activity (micromoles of substrate transformed/mg protein/min)	Reduction in enzyme activity after ultraviolet exposure
$(Na^+ + K^+)$-ATPase	3·02 ± 0·24	20·1 ± 3·7
Mg^{2+}-ATPase	0·33 ± 0·06	17·5 ± 4·0
Cholinesterase	0·083 ± 0·009	13·3 ± 2·3
NADH-cytochrome *c* reductase	0·110 ± 0·015	18·1 ± 1·3

* Microsomal fractions were prepared and the enzyme activities were assayed at 37°. Per cent inhibition of the enzyme activities was calculated and compared with the activities of the same microsomal preparation assayed without ultraviolet exposure. Mean ± S.E. of eight experiments.

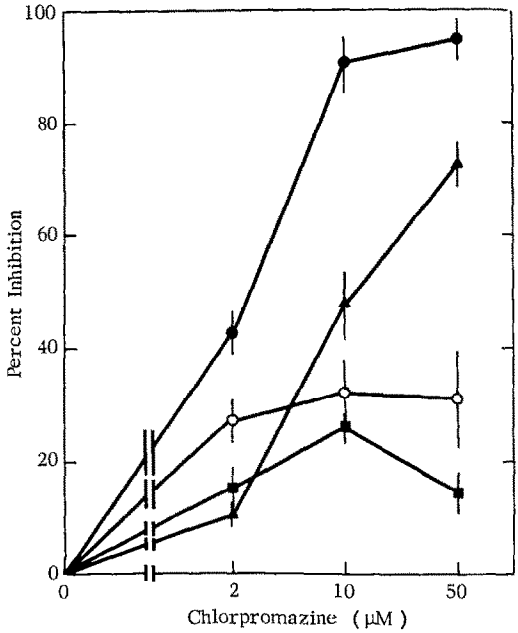

FIG. 2. Effect of chlorpromazine on microsomal enzyme activities after ultraviolet exposure of the chlorpromazine–microsome mixture. Same as Fig. 1, except that the chlorpromazine–microsome mixture was exposed to ultraviolet light at 25° for 4 min instead of storing in absence of light prior to enzyme assay. Control enzyme preparations were also exposed to ultraviolet light in the absence of chlorpromazine. ●——● $(Na^+ + K^+)$-ATPase. ○——○ Mg^{2+}-ATPase. ▲——▲ Cholinesterase. ■——■ NADH-cytochrome *c* reductase.

The concentration of chlorpromazine free radical could not be determined because of the low steady state concentration even during ultraviolet exposure.[1,2]

There was a considerable difference in the sensitivity of four microsomal enzyme activities to chlorpromazine after the ultraviolet exposure (Fig. 2). $(Na^+ + K^+)$-ATPase activity was most sensitive. Cholinesterase activity required approximately a 5-fold increase in concentration of chlorpromazine for a similar inhibition. With Mg^{2+}-ATPase and NADH-cytochrome *c* reductase activities, somewhat different concentration–response curves were obtained. NADH-cytochrome *c* reductase activity was least sensitive among the microsomal enzyme activities studied, and enhancement of chlorpromazine inhibition following ultraviolet exposure was minimal.

Removal of oxygen from the chlorpromazine–microsome mixture during ultraviolet exposure, as well as in the non-exposed mixture, had little effect on the inhibition of $(Na^+ + K^+)$-ATPase activity (Table 2), indicating that the inhibition of the enzyme activity was not influenced by the presence of molecular oxygen.

The inhibition of $(Na^+ + K^+)$-ATPase activity by chlorpromazine free radical increased progressively with time (Table 3). Since the assay of $(Na^+ + K^+)$-ATPase activity required a 15-min incubation period, the values obtained were calculated from the average reaction velocity during such an incubation period at 37°. After longer preincubation periods however, such average reaction velocities were lower, indicating that the reaction velocity decreased with time. A similar pattern was observed with cholinesterase activity (Fig. 3). In this case, the reaction was monitored

TABLE 2. EFFECT OF ULTRAVIOLET EXPOSURE ON CHLORPROMAZINE INHIBITION OF MICROSOMAL $(Na^+ + K^+)$-ATPASE ACTIVITY UNDER AEROBIC AND ANAEROBIC CONDITIONS*

Gas phase	Ultraviolet exposure	Per cent inhibition (mean ± S.E.)
Air		−2·5 ± 3·9
N_2		4·2 ± 2·5†
Air	+	61·5 ± 6·1
N_2	+	58·4 ± 4·5†

* A medium containing 10 μM chlorpromazine and 50 μg of microsomal protein in 2·5 ml of 100 mM tris–HCl buffer, pH 7·4, was exposed to ultraviolet light under aerobic or anaerobic conditions. Mixtures which were not exposed to ultraviolet light were kept in a dark place for a comparable period. Per cent inhibition of the $(Na^+ + K^+)$-ATPase activity was calculated against controls in which chlorpromazine was eliminated.

† Not different from aerobic values either by the group or paired *t*-test.

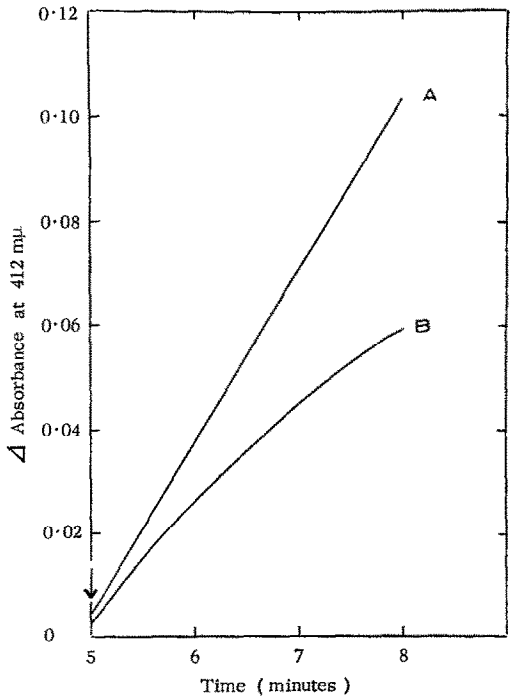

FIG. 3. Inhibition of microsomal cholinesterase activity by ultraviolet exposure of the chlorpromazine–microsome mixture. A mixture containing 50 μg of microsomal protein in 2·5 ml of 100 mM tris–HCl buffer (pH 7·5) with (curve B) or without (curve A) 10 μM chlorpromazine added to phosphate buffer and DTNB reagent. After a 5-min preincubation period at 37°, 20 μl of 0·75 M acetylthiocholine was added to the sample cuvette as indicated by the arrow. The reference cuvette contained the same mixture, and 20 μl of water was added instead of acetylthiocholine. Figure is recording spectrophotometer trace of a typical experiment.

continuously. The velocity of the chlorpromazine-inhibited reaction decreased during the incubation period (Fig. 3, curve B).

TABLE 3. EFFECT OF PREINCUBATION TIME ON THE INHIBITION OF $(Na^+ + K^+)$-ATPASE ACTIVITY BY CHLORPROMAZINE FREE RADICAL*

Preincubation period (min)	Inhibition of $(Na^+ + K^+)$-ATPase activity (%)
3	61·0 ± 7·5
5	60·7 ± 5·9
20	73·5 ± 6·6†
35	78·6 ± 7·2†

* Conditions same as in Fig. 2. Chlorpromazine concentration was 10 μM.

† Statistically different from 3-min value ($P < 0.05$). Mean ± S.E. of five experiments.

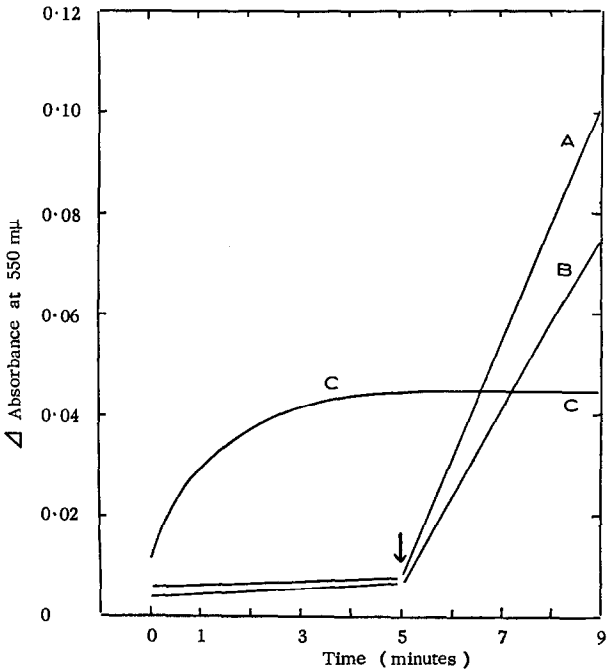

FIG. 4. Inhibition of microsomal NADH-cytochrome *c* reductase activity by the ultraviolet exposure of the chlorpromazine–microsome mixture. A mixture containing 50 μg of microsomal protein in 2·5 ml of 100 mM tris–HCl buffer (pH 7·5) with (curve B) or without (curve A) 10 μM chlorpromazine was exposed to ultraviolet light at 25° for 4 min and KCN and cytochrome *c* solutions (time "0") immediately added. After a 5-min preincubation period at 37°, 0·1 ml of 1·15 mM NADH was added to the sample cuvette as indicated by the arrow. The reference cuvette contained the same mixture, and 0·1 ml of water was added instead of NADH. Curve C: after the ultraviolet exposure of the chlorpromazine in 100 mM tris–HCl buffer (pH 7·5), KCN and cytochrome *c* solutions were added to only reference cuvette (time "0"), and the absorbance at 550 mμ was recorded against water. Neither microsomal preparation nor NADH was added. Figure is recording spectrophotometer trace of a typical experiment.

With NADH-cytochrome *c* reductase activity, neither the velocity of the chlorpromazine free radical-inhibited reaction (Fig. 4, curve B) nor that of the control reaction (Fig. 4, curve A) decreased with time. There was an interaction between the chlorpromazine free radical and ferricytochrome *c*. As can be seen from curve C of Fig. 4, chlorpromazine free radical reduced ferricytochrome *c* in the absence of the microsomal preparation and NADH. The reduction of ferricytochrome *c* terminated approximately 4 min after ultraviolet exposure regardless of the chlorpromazine concentration. This interaction was also observed in the presence of microsomal protein, although it was attenuated markedly. The effect of such an interaction was eliminated from the experiment shown in curve B of Fig. 4 by placing the same ultraviolet exposed chlorpromazine–microsome mixture and cytochrome *c* in the reference cuvette. Removal of oxygen from the chlorpromazine solution prior to the ultraviolet exposure had a minimal effect on this interaction even after vigorous bubbling of the solution with a nitrogen stream.

DISCUSSION

A study of chlorpromazine free radical–enzyme interaction has several limitations. First, the inhibitor concentration is not known.[1,2] In the present paper, the drug concentration refers to the concentration of chlorpromazine initially added to the mixture, although the actual inhibitory species was the free radical and not chlorpromazine itself. It is assumed that the free radical was generated in proportion to the initial chlorpromazine concentration during the ultraviolet exposure. However, it was possible to compare the inhibition of various enzyme activities after the treatment.

Second, the inhibited reaction was not linear with time in the estimation of both $(Na^+ + K^+)$-ATPase and cholinesterase activities. Thus, the per cent inhibition of the enzyme activities shown in Fig. 2 represents the average inhibition during the assay period. The time course of Mg^{2+}-ATPase inhibition was less clear due to large variability. The assay conditions for ATP-ase activity were optimal for the more active $(Na^+ + K^+)$-ATPase activity, and this resulted in a larger experimental error in the assay of the lower Mg^+-ATPase activity.

Among the three enzyme activities compared for time course of the inhibition, progressive inhibition was observed only with those enzyme systems which were highly sensitive such as $(Na^+ + K^+)$-ATPase and cholinesterase, whereas the inhibition of the less sensitive NADH-cytochrome *c* reductase activity did not progress with time. Therefore, it is conceivable that the difference in inhibitory effects may be much smaller during or immediately after the ultraviolet exposure of the chlorpromazine–microsome mixture. The progression of the inhibition is not due to the further interaction with free radical in the mixture since no free radical is present in the medium more than 4 min after ultraviolet exposure. In the presence of protein, the existence of the chlorpromazine free radical is much shorter than 4 min.[2]

A reduction of cytochrome *c* by chlorpromazine free radical was observed. This reaction is non-enzymatic and is terminated within 4 min after ultraviolet exposure regardless of the chlorpromazine concentration added to the mixture. It appears that the termination of this reaction is governed by spontaneous decay of chlorpromazine free radical. McCord and Fridovich[22] have demonstrated that certain electron carriers mediate the reduction of cytochrome *c* through superoxide anion radicals. However, the reduction of cytochrome *c* in the presence of chlorpromazine free radical does

not involve the superoxide anion radicals since the rate and the magnitude of the reaction was minimally influenced by anaerobic conditions. This may be in agreement with the previous observation by McCord and Fridovich[22] who observed that the reduction of cytochrome *c* does not proceed through the superoxide anion radicals when the electron carrier is methylene blue, a compound similar to chlorpromazine.

It is also well known that the photo-oxidation of proteins may be sensitized in the presence of "dyes" (see Foote).[23] That the inhibition of the $(Na^+ + K^+)$-ATPase produced by ultraviolet exposure of the chlorpromazine–enzyme mixture does not involve "excited" oxygen is shown in the present studies in which the enhancement of the chlorpromazine inhibition of $(Na^+ + K^+)$-ATPase by ultraviolet exposure was not affected by the removal of oxygen from the chlorpromazine–enzyme mixture during ultraviolet exposure. The reduction of cytochrome *c* by chlorpromazine free radical did not interfere with the present enzyme assay, since all enzyme activities were assayed after a 5-min preincubation period. The amount of cytochrome *c* reduced by chlorpromazine free radical was not too large to limit the subsequent enzymatic reduction. Furthermore, any reduction of cytochrome *c* which was not due to the NADH-cytochrome *c* oxido-reduction reaction was cancelled out by placing the same ultraviolet exposed chlorpromazine–microsomal mixture in the reference cuvette. The reduction of cytochrome *c*, with chlorpromazine as an electron donor, was not observed even in the presence of the microsomal preparation.*

It has been previously shown that reagents with highly reactive sulfhydryl groups, such as dithiothreitol or cysteine, reactivate $(Na^+ + K^+)$-ATPase inhibited by chlorpromazine free radical,[2] perhaps by removing the enzyme-bound inhibitor. Thus, it appears that the various binding sites for chlorpromazine free radical have different affinities. During ultraviolet exposure, the free radical seems to bind to these sites rather indiscriminately and subsequently redistributes to sites with higher affinities.

Sulfhydryl groups on $(Na^+ + K^+)$-ATPase have been shown to be an inhibitory site for chlorpromazine free radical.[7] NADH-cytochrome *c* reductase is known to contain reactive sulfhydryl groups,[12] although the enzyme activity was rather insensitive to chlorpromazine free radical. In a previous paper, we have shown that 25 per cent of the sulfhydryl groups contained in a partially purified $(Na^+ + K^+)$-ATPase preparation were unaffected when the enzyme activity was inhibited completely by chlorpromazine free radical.[7] Thus, it appears that various sulfhydryl groups have different affinities for chlorpromazine free radical.

The inhibition of the $(Na^+ + K^+)$-ATPase activity observed after the ultraviolet exposure of the chlorpromazine–enzyme mixture has been shown to be the result of the free radical bound to the enzyme.[2] This, however, may not be true for the inhibition of the other enzyme activities. It is possible that photo-oxidation of the enzyme protein sensitized by chlorpromazine, or denaturation of the enzyme protein, may be the mechanism of the reduction of the enzyme activity. Nevertheless, the present study clearly indicates that there is some selectivity in the inhibitory effect of chlorpromazine free radical on microsomal enzyme activities in that Mg^{2+}-ATPase, cholinesterase and NADH-cytochrome *c* reductase were less sensitive to chlorpromazine free radical than $(Na^+ + K^+)$-ATPase.

Acknowledgements—We thank Mrs. J. Cline and Mr. Hip-Man So for technical assistance.

* T. Akera and T. M. Brody, unpublished observation.

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