# The Interaction between Chlorpromazine Free Radical and Microsomal Sodium- and Potassium-Activated Adenosine Triphosphatase from Rat Brain

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SUMMARY

The inhibitory effect of chlorpromazine free radical on $(Na^+ + K^+)$-ATPase [$Mg^{2+}$-dependent, $(Na^+ + K^+)$-activated ATP phosphohydrolase] activity was studied by means of ultraviolet irradiation to generate the free radical, with a deoxycholate- and NaI-treated microsomal fraction obtained from rat brain as the enzyme source. Treatment with NaCl, cysteine, or dithiothreitol restored the activity of the enzyme preparation inhibited by chlorpromazine free radical. Washing of the inhibited enzyme preparation with Tris-HCl buffer or addition of sodium metabisulfite or of ascorbic acid to the incubation mixture did not restore the activity of the inhibited enzyme preparation. It appeared that the inhibition by chlorpromazine free radical was of either the "pseudo-irreversible" or the irreversible type. Inhibition of the enzyme activity by chlorpromazine free radical and *p*-hydroxymercuribenzoate was reduced, while inhibition by ouabain was enhanced, at low KCl concentrations. These data indicate that the mechanism by which chlorpromazine free radical and *p*-hydroxymercuribenzoate inhibit the $(Na^+ + K^+)$-ATPase system is different from that of ouabain. $K^+$-dependent *p*-nitrophenyl phosphatase activity was as sensitive to the inhibitory effect of chlorpromazine free radical as $(Na^+ + K^+)$-ATPase activity, while $Mg^{2+}$-dependent ATPase activity in the same preparation was significantly less sensitive.

## INTRODUCTION

Chlorpromazine has been shown to inhibit the transport of a variety of substances across cell membranes (1). Consequently, several authors (2–6) have attempted to demonstrate inhibition of a $(Na^+ + K^+)$-ATPase [$Mg^{2+}$-dependent, $(Na^+ + K^+)$-activated ATP phosphohydrolase] by chlorpromazine, since accumulating evidence indicates that this enzyme system is related to active transport across cell membranes (7).

Most studies, however, have indicated that the $(Na^+ + K^+)$-ATPase is rather insensitive to chlorpromazine. The majority of investigators have reported that 0.1 mM chlorpromazine inhibited less than 40% of the enzyme activity *in vitro*, although Squires (4) observed about 90%, or almost complete, inhibition when the enzyme and the inhibitor were first incubated together for 30 min at 37° in the absence of added NaCl or KCl.

Recently, we have found that a semiquinone free radical of chlorpromazine is a potent inhibitor of brain microsomal $(Na^+ + K^+)$-ATPase activity and that the free radical form of chlorpromazine, rather than chlorpromazine itself, is responsible for the inhibition of the enzyme system *in vitro* (8).

The highly reactive free radical form of

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chlorpromazine may also have a functional role, since it can be produced *in vivo* during the oxidative metabolism of chlorpromazine (9), by interaction of chlorpromazine with manganese (10) or melanin (11, 12), or even by the exposure of the eye to light. Eye complications are among the most frequent side effects observed during long-term chlorpromazine therapy (13).

The present report compares the kinetic behavior of chlorpromazine free radical with respect to microsomal $(Na^+ + K^+)$-ATPase obtained from rat brain with that of other known inhibitors of the enzyme, ouabain and *p*-hydroxymercuribenzoate. The effects of chlorpromazine free radical on the activities of a $(Na^+ + K^+)$-ATPase and $K^+$-dependent *p*-nitrophenyl phosphatase present in the deoxycholate- and NaI-treated microsomal fraction are also compared.

## METHODS

Male Sprague-Dawley rats weighing 200–300 g were used. The preparation of the enzyme from the microsomal fraction and the assay of ATPase activity were performed as reported previously (8). In the present studies, the deoxycholate-treated microsomal fraction was further treated with NaI, using the method of Nakao *et al.* (14) with some modifications. Following the last centrifugation in the protocol described previously (8), the microsomal pellet was suspended in a mixture containing 2.0 M NaI, 2.5 mM disodium EDTA, 3.0 mM $MgCl_2$, 3.0 mM disodium ATP, and 5.0 mM histidine HCl adjusted to pH 7.3 with Tris base. The suspension was stirred gently for 30 min at 0°, diluted with water to 0.8 M NaI, and centrifuged for 30 min at 100,000 $\times g$. The resulting pellet was washed twice by suspending it in 10 mM Tris-HCl buffer (pH 7.3) and centrifuging it at 100,000 $\times g$ for 30 min. The final residue was suspended in a medium containing 0.25 M sucrose, 5.0 mM histidine HCl, and 1.0 mM Tris-EDTA (pH 7.0). The preparation was stored frozen until use. Protein was assayed by the method of Lowry *et al.* (15), using bovine serum albumin as a standard.

All incubations were performed in the dark. Unless otherwise stated, $Mg^{2+}$-ATPase ($Mg^{2+}$-dependent ATP phosphohydrolase) activity was subtracted from the total ATPase activity assayed in the presence of NaCl, KCl, and $MgCl_2$ to calculate the $(Na^+ + K^+)$-ATPase activity. Since the enzyme preparation added to each incubation tube (16 $\mu$g of enzyme protein in a total incubation volume of 1.0 ml) did not contain a measurable amount of inorganic phosphate, a blank from which the enzyme preparation was omitted was employed to calculate the $Mg^{2+}$-ATPase activity. Preliminary experiments indicated that there was no difference between these values and those obtained by incubation of the enzyme preparation with ATP and buffer in the absence of added $MgCl_2$ or from those obtained from "zero time" incubations.

$K^+$-activated *p*-nitrophenyl phosphatase activity was assayed by using 5.0 mM disodium *p*-nitrophenyl phosphate as the substrate instead of Tris-ATP and omitting NaCl from the incubation mixture for the ATPase assay. Since preliminary experiments indicated that up to 10 mM NaCl has little effect on $K^+$-activated *p*-nitrophenyl phosphatase activity in either the presence or absence of 15 mM KCl, no effort was made to remove sodium from the substrate. The enzyme activity was estimated by measuring the *p*-nitrophenol liberated from *p*-nitrophenyl phosphate as described by Inturrisi and Titus (16), using authentic *p*-nitrophenol (spectrophotometric grade, Sigma Chemical Company) as a standard.

When the chlorpromazine-enzyme mixture was exposed to ultraviolet light in order to generate the free radical of chlorpromazine (8), the drug and the enzyme preparation were in a solution containing 100 mM Tris-HCl buffer (pH 7.5). This mixture was placed in a quartz cuvette with a 1.0-cm light path (or, when the volume was large, a shallow beaker was used and exposed from above) and exposed to ultraviolet light for 4 min at 23–25°. A mineralight model R-51 lamp (primary emission wavelength, 253.7 m$\mu$), with its filter removed, was used as an ultraviolet light source, and the samples were placed 45 cm from the lamp. After ultraviolet exposure, the mixture was diluted 2-fold with solu-

tions containing the necessary ions and substrate.

Spectrophotometric analysis revealed that under these conditions approximately 35% of the added chlorpromazine was degraded by ultraviolet irradiation. Histidine, a component of the enzyme suspension medium, was maintained at 2.0 mM during the ultraviolet exposure, since histidine had a protective effect on the enzyme activity during ultraviolet irradiation under these experimental conditions.

Since the estimation of the chlorpromazine free radical concentration was not possible (8), the drug concentrations refer to the molar concentrations of chlorpromazine calculated from the amount of the drug initially added to the mixture. It should be noted, however, that the actual inhibitory species is the chlorpromazine free radical. In most instances, the concentration of chlorpromazine present in the incubation mixture during the ATPase assay was lower than that indicated, since a significant portion of chlorpromazine was degraded by ultraviolet irradiation.

The semiquinone free radical of chlorpromazine in the solid state was prepared by a procedure which is essentially a modification of the methods of Felmeister *et al.* (17) and Levy and Burbridge (18). Equimolar amounts of chlorpromazine HCl (17.75 mg) and chlorpromazine sulfoxide HCl (18.75 mg) were dissolved in 1.0 ml of 70% perchloric acid and stirred for 5 min at 25°. The solution was chilled to about 0° and added to 0.5 ml of acetone, followed by approximately 20 ml of ether to yield two distinct layers. The mixture was kept at 0°, stirred for 15 min, and then filtered through a Thomas fused glass filter (medium porosity). The dark red residue, the free radical of chlorpromazine, was washed several times with ether and dried at room temperature. The material was used without further purification and, if kept in a dark, dry environment, was stable for several months.

The absorption spectra of chlorpromazine and chlorpromazine free radical were recorded with a Shimadzu model MPS-50L recording spectrophotometer. The concentration of chlorpromazine free radical was calculated from the absorbance at 527 mμ, assuming the molar extinction coefficient to be 10 $mM^{-1}$ $cm^{-1}$ (8).

The decay of chlorpromazine free radical was studied in the following manner. To a test tube containing solid state free radical of chlorpromazine, a 50 mM Tris-HCl buffer solution (pH 5.8) with or without 4.0 μg of the enzyme protein per milliliter was added to make the initial concentration of chlorpromazine free radical 100 μM. After rapid, vigorous mixing, the mixture was transferred to a cuvette and placed in the sample chamber of a Shimadzu model MPS-50L spectrophotometer. The wavelength was set at 527 mμ, and the absorbance was recorded continuously.

The results of the enzyme assay were analyzed for significance by Student's *t*-test for group comparison.

## RESULTS

*Reversibility of inhibition of microsomal $(Na^+ + K^+)$-ATPase activity by chlorpromazine free radical.* Initial studies were performed to demonstrate the possible reversibility of the chlorpromazine free radical-induced inhibition of the enzyme activity. Buffer or NaCl washes were used to attempt to restore activity to control levels. The control ATPase activity of the NaI-treated microsomal fraction assayed in the presence of 100 mM NaCl, 15 mM KCl, 5.0 mM $MgCl_2$, and 5.0 mM Tris-ATP was $44 \pm 2.6$ μmoles of inorganic phosphate released per milligram of protein in 10 min (mean of 11 enzyme preparations ± standard error). The $Mg^{2+}$-ATPase activity of the same enzyme preparations was $3.7 \pm 0.4$. Ultraviolet light exposure of the enzyme preparations, in the absence of chlorpromazine, resulted in a slight reduction of enzyme activity (approximately 6%; statistically not significant). Treatment of the inhibited enzyme preparation with 1.0 M NaCl restored $(Na^+ + K^+)$-ATPase activity reduced by ultraviolet irradiation of the chlorpromazine-enzyme mixture, as shown in Fig. 1. There is a significant difference ($p < 0.01$) between the $(Na^+ + K^+)$-ATPase activity observed after NaCl treat-

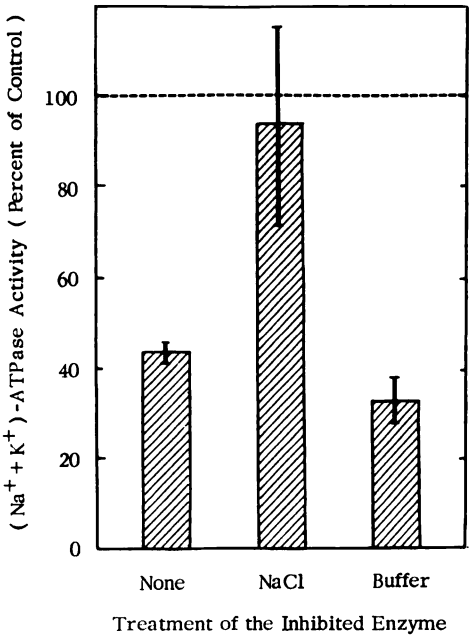


FIG. 1. *Effect of NaCl and buffer treatment on the microsomal ($Na^+ + K^+$)-ATPase activity inhibited by chlorpromazine free radical*

The enzyme preparation (16 μg of protein per milliliter) was exposed to ultraviolet light in the presence of 6 μM chlorpromazine in a total volume of 100 ml to obtain an inhibited enzyme preparation. *Left:* "none" indicates that the ATPase activity of the inhibited enzyme preparation was assayed immediately after ultraviolet exposure. *Center* (NaCl treatment): the inhibited enzyme preparation was centrifuged at 40,000× *g* for 30 min, and the pellet was resuspended in 1.0 M NaCl (pH 7.5) and incubated at 37° for 30 min. The mixture was then washed three times by centrifugation at 100,000 × *g* for 30 min and resuspension with a solution containing 0.32 M sucrose, 5.0 mM histidine, and 1.0 mM Tris-EDTA (pH 7.0, adjusted with Tris base). *Right* (buffer treatment): the procedure was same as for NaCl treatment, except that 50 mM Tris-HCl buffer (pH 7.5) was used instead of 1.0 M NaCl. The ATPase activity of these preparations was compared with that of a control preparation treated in the same manner after ultraviolet irradiation in the absence of chlorpromazine. Each value represents the mean of seven experiments. Vertical lines indicate the standard error of the mean.

ment of the inhibited enzyme and that observed immediately after ultraviolet light exposure of the drug-enzyme mixture (labeled "none" on the abscissa). After treatment of the inhibited enzyme with 50 mM Tris-HCl buffer (pH 7.5), the activity was similar to that observed immediately after ultraviolet irradiation. Thus, no reversal was apparent after washing the inhibited enzyme with Tris-HCl buffer.

The addition of cysteine and dithiothreitol (*threo*-2,3-dihydroxy-1,4-dithiobutane) to the incubation mixture restored, at least partially, the enzyme activity inhibited by chlorpromazine free radical, as can be seen in Fig. 2, where percentage inhibition of the enzyme activity is plotted against the

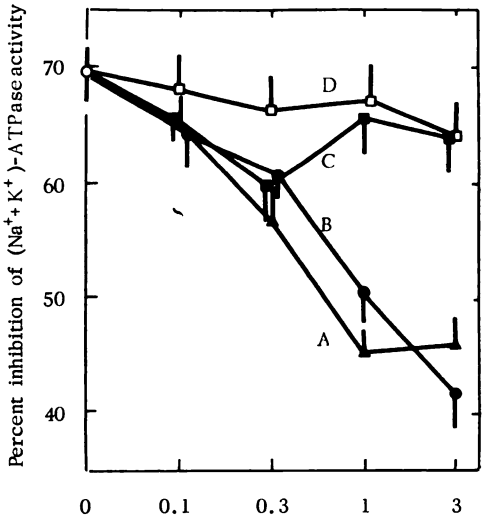


FIG. 2. *Effect of reducing agents on the ($Na^+ + K^+$)-ATPase activity inhibited by chlorpromazine free radical*

The enzyme preparation (16 μg of protein per milliliter) was exposed to ultraviolet light in the presence of 10 μM chlorpromazine to form the free radical. The inhibited enzyme preparation was then added to an incubation mixture containing cysteine (*A*), dithiothreitol (*B*), sodium metabisulfite (*C*), or ascorbic acid (*D*). Ascorbic acid was adjusted to pH 7.5 with Tris solution. After a 15-min incubation at 37°, Tris-ATP was added to start the ATPase reaction. The ($Na^+ + K^+$)-ATPase activity was compared with that of the enzyme preparation exposed to ultraviolet light in the absence of chlorpromazine. Each point represents the mean of five experiments. Vertical lines indicate one standard error of the mean. None of these reagents had any effect on uninhibited ($Na^+ + K^+$)-ATPase activity at the concentrations employed.

concentrations of these agents. Two other reducing agents, sodium metabisulfite and ascorbic acid, were ineffective. Thus, the ($Na^+ + K^+$)-ATPase activity inhibited by chlorpromazine free radical can be "reactivated" (19), although the reversibility is not easily demonstrable. These studies also indicate that chlorpromazine free radical does not denature the enzyme.

An attempt was made to determine whether the drug-enzyme interaction was "pseudo-irreversible" (or irreversible) by plotting percentage inhibition as a function of enzyme concentration. A pseudo-irreversible or irreversible inhibitor would give a parabolic curve, representing lower inhibition at higher protein concentrations, while reversible inhibitors would produce a straight line parallel to the enzyme concentration axis (20). Analysis was not entirely successful, probably because of the lower incidence of free radical-enzyme interaction at lower enzyme concentrations; i.e., more free radical was disproportionated before reacting with protein. Analysis was also limited because of the difficulty of increasing the protein concentration beyond certain levels. In the presence of more than 100 μg of microsomal protein per milliliter, the amount of chlorpromazine degraded by standard ultraviolet irradiation decreased. However, significantly greater inhibition was observed at lower protein concentrations, an observation which would be consistent with irreversible or pseudo-irreversible inhibition.

*Rate of reaction of chlorpromazine free radical with the ($Na^+ + K^+$)-ATPase preparation.* In order to determine the rate of reaction between the chlorpromazine free radical and the enzyme preparation, the decay of chlorpromazine free radical in the presence and absence of the enzyme preparation was studied by continuously monitoring absorbance at 527 mμ. At pH 7.5, where all enzymatic studies were carried out, the decay of chlorpromazine free radical was so rapid that accurate measurement of decay kinetics was not possible. In Fig. 3 the reciprocal of chlorpromazine free radical concentration is plotted against time. When chlorpromazine free radical was allowed to disproportionate at 30° in 50 mM Tris-HCl buffer at pH 5.8, where the rate of disproportionation is slower, this plot yielded a straight line (curve *A*). This would indicate that the disproportionation reaction follows second order kinetics (17), as in the following equations.

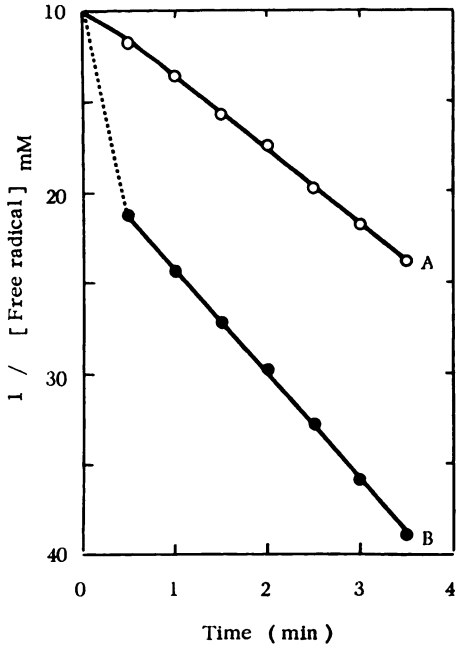


FIG. 3. *Disproportionation of chlorpromazine free radical at pH 5.8*

The reciprocal of the chlorpromazine free radical concentration, calculated from the absorbance at 527 mμ, was plotted against the time elapsed after the addition of 50 mM Tris-HCl buffer, pH 5.8 (*A*), or a solution containing 4.0 μg of NaI-treated microsomal protein per milliliter of Tris-HCl buffer (*B*) to the solid state chlorpromazine free radical. The reaction was carried out at 30°.

$$R + R \xrightarrow{K} \text{chlorpromazine} + \text{phenazathionium ion}$$

$$\text{Decay velocity} = \frac{d[R]}{dt} = -K[R]^2$$

Thus

$$\frac{1}{[R]} = \frac{1}{[R]_0} + Kt$$

where $R$ is chlorpromazine free radical, $[R]$ is its concentration at time $t$, and $[R]_0$ is its initial concentration.

The $K$ value calculated from the slope was 4.1 mM$^{-1}$ min$^{-1}$. The presence of 4.0 $\mu$g of NaI-treated microsomal protein per milliliter of Tris-HCl increased only the initial rate of decay of chlorpromazine free radical (curve $B$). The first observation was made 0.5 min after mixing. By this time, the reaction between chlorpromazine free radical and the enzyme preparation was complete. The apparent $K$ value calculated for this period was 22.6 mM$^{-1}$ min$^{-1}$. After 0.5 min, the rate of decay ($K$ = 5.9 mM$^{-1}$ min$^{-1}$) was not significantly different from the value estimated in the absence of the enzyme preparation. Four different estimations of the apparent $K$ value for the above equations at pH 7.5 ranged from 60 to 230 mM$^{-1}$ min$^{-1}$, with a mean value of 135 mM$^{-1}$ min$^{-1}$, in the absence of enzyme protein. Thus, the initial phase of curve $B$ illustrates the very rapid interaction of chlorpromazine free radical with the enzyme protein.

*Effect of chlorpromazine free radical on microsomal ($Na^+ + K^+$)-ATPase activity at various potassium concentrations.* The effect of chlorpromazine free radical on the kinetic behavior of ($Na^+ + K^+$)-ATPase is shown in Fig. 4, where the reciprocal of the rate of hydrolysis is plotted against the reciprocal of the KCl concentration. As the concentration of chlorpromazine, and therefore the concentration of chlorpromazine free radical, is increased, the apparent $K_m$ (Michaelis constant) and maximum velocity of the inhibited reaction decrease, resulting in a set of curves suggestive of so-called "uncompetitive" inhibition (19) with respect to KCl.

This is shown more clearly in Fig. 5, where $\alpha(1-\alpha)^{-1}$ $[I]$ is plotted against the concentration of KCl according to the method of Hunter and Downs (21). $\alpha$ represents the fractional (or inhibited) activity, and $[I]$ is the concentration of chlorpromazine. Note that the concentrations of chlorpromazine in the figure are those initially added to the mixture and do not represent the concentration of chlorpromazine free radical or that of chlorpromazine during the incubation period. The inhibition observed, however, is caused by the free radical of chlorpromazine. The effects of ouabain and $p$-hydroxymercuribenzoate, both known inhibitors of ($Na^+ + K^+$)-ATPase activity (7), are also compared.

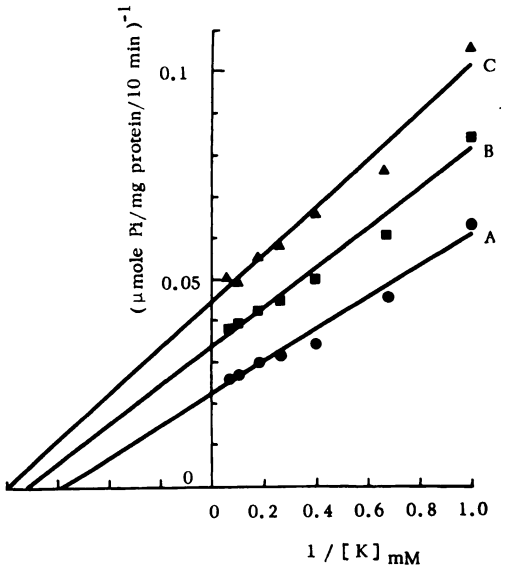


FIG. 4. *Effect of chlorpromazine free radical on microsomal ($Na^+ + K^+$)-ATPase activity*

Double-reciprocal (Lineweaver-Burk) plot of ($Na^+ + K^+$)-ATPase activity against KCl concentration. The chlorpromazine-enzyme mixture was exposed to ultraviolet light prior to ATPase assay. The ATP concentration was 5.0 mM in the presence of 5.0 mM $MgCl_2$ and 100 mM NaCl with 16 $\mu$g of enzyme protein in a final incubation volume of 1.0 ml. Chlorpromazine concentrations in the final incubation volume were calculated from the amount of chlorpromazine initially added to the mixture and were 0 ($A$), 1.0 ($B$), or 2.0 ($C$) $\mu$M. Although the actual inhibitor was chlorpromazine free radical, the data refer to the chlorpromazine concentration (see METHODS). Each point represents the mean of five experiments. Linear regression lines were fitted by the method of least squares.

The curve obtained with $p$-hydroxymercuribenzoate as an inhibitor was typical of uncompetitive inhibition,[1] similar to that obtained with chlorpromazine free radical, while the curve obtained with ouabain as the inhibitor was indicative of competitive inhibition at low KCl concentration and noncompetitive inhibition at higher KCl concentrations.

[1] This type of inhibition is sometimes referred to as "coupling" inhibition [see Webb (20)].

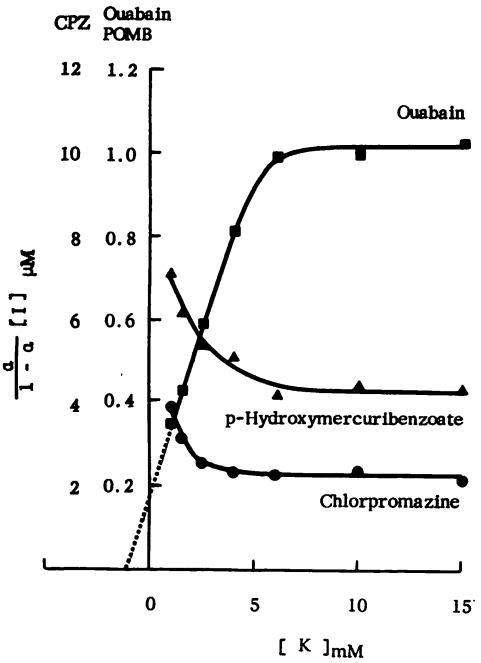


FIG. 5. *Comparison of the effects of chlorpromazine free radical (CPZ), ouabain, and p-hydroxymercuribenzoate (POMB) on microsomal $(Na^+ + K^+)$-ATPase activity*

Hunter-Downs plot. The chlorpromazine-enzyme mixture was exposed to ultraviolet light prior to the ATPase assay. Ouabain and *p*-hydroxymercuribenzoate were added to an unexposed enzyme preparation. The ATPase assay was carried out after a 5-min incubation at 37° with various KCl concentrations. Fractional activity ($\alpha$) of the enzyme preparation inhibited by chlorpromazine free radical was calculated against the activity of the enzyme preparation exposed to ultraviolet light in the absence of chlorpromazine. The ATP concentration was 5.0 mM in the presence of 5.0 mM $MgCl_2$ and 100 mM NaCl. The concentrations of inhibitors were: chlorpromazine, 2.0 $\mu$M; ouabain, 0.3 and 1.0 $\mu$M; and *p*-hydroxymercuribenzoate, 1.0 $\mu$M, with 16 $\mu$g of enzyme protein in a final incubation volume of 1.0 ml. Although the inhibition is due to chlorpromazine free radical, the data refer to chlorpromazine concentrations in the final incubation volume calculated from the amount of the drug initially added to the mixture (see METHODS). Each point represents the mean of five experiments.

*Effects of chlorpromazine free radical on microsomal $(Na^+ + K^+)$-ATPase, $K^+$-p-nitrophenyl phosphatase, and $Mg^{2+}$-ATPase activities.* The sensitivities of microsomal $(Na^+ + K^+)$-ATPase, $K^+$-activated *p*-nitrophenyl phosphatase, and $Mg^{2+}$-ATPase activities to the inhibitory effect of chlorpromazine free radical are shown in dose-response curves plotted in Fig. 6, where the percentage inhibition of the enzyme activities is plotted against the concentration of

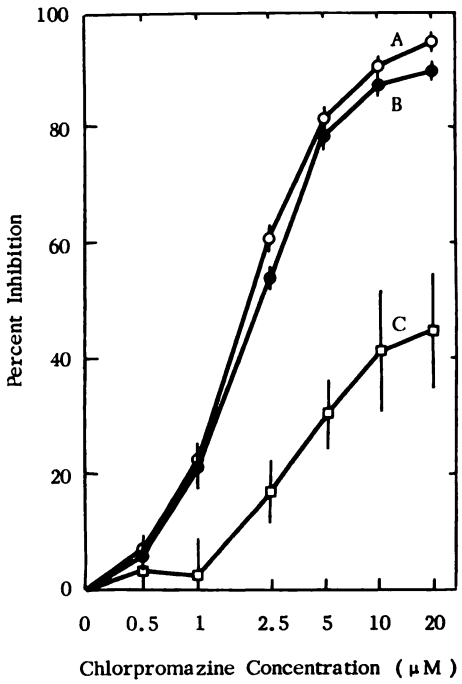


FIG. 6. *Effects of chlorpromazine free radical on microsomal ATPase and $K^+$-activated p-nitrophenyl phosphatase activities*

The chlorpromazine-enzyme mixture was exposed to ultraviolet light prior to assay of enzyme activity. After 5 min of incubation at 37°, substrates were added to start the reaction. The activity was compared with that of each control enzyme preparation exposed to ultraviolet light in the absence of added chlorpromazine. The concentrations during the incubation were: $MgCl_2$, 5.0 mM; Tris-ATP or *p*-nitrophenyl phosphate, 5.0 mM; with or without 100 mM NaCl and/or 15 mM KCl. The concentrations of chlorpromazine in the final incubation volume of 1.0 ml were calculated from the amount which was added initially to the mixture and do not indicate the actual chlorpromazine concentrations during the incubation. *A*, $(Na^+ + K^+)$-ATPase; *B*, $K^+$-*p*-nitrophenyl phosphatase; *C*, $Mg^{2+}$-ATPase activities. Each point represents the mean of five experiments. Vertical lines indicate the standard error of the mean.

chlorpromazine. In these studies, it was assumed that the fraction of chlorpromazine converted to the free radical during ultraviolet irradiation was the same irrespective of the initial chlorpromazine concentration. The relative sensitivity of these three enzyme systems would not be affected by these differences, since aliquots of the same chlorpromazine-enzyme mixture were assayed for different enzyme activities after exposure to ultraviolet light.

Chlorpromazine free radical inhibited the microsomal $(Na^+ + K^+)$-ATPase and $K^+$-activated *p*-nitrophenyl phosphatase activities to the same extent. The $Mg^{2+}$-ATPase activity of the same preparation, however, was less sensitive to the chlorpromazine free radical than were the $(Na^+ + K^+)$-ATPase and $K^+$-activated *p*-nitrophenyl phosphatase activities. Furthermore, only about 50% of the $Mg^{2+}$-ATPase activity could be inhibited by relatively high concentrations of chlorpromazine free radical. The large variation in the $Mg^{2+}$-ATPase activity resulted from the relatively low enzyme activity. Conditions of these studies were optimal for the highly active $(Na^+ + K^+)$-ATPase activity, and thus a larger error in the estimation of the less active $Mg^{2+}$-ATPase activity was observed. While approximately 50% of the $Mg^{2+}$-ATPase activity was inhibited by high concentrations of chlorpromazine free radical, this enzyme activity was unaffected by up to 0.1 mM ouabain.

The concentration of chlorpromazine required for 50% inhibition ($I_{50}$) of the microsomal $(Na^+ + K^+)$-ATPase and $K^+$-activated *p*-nitrophenyl phosphatase activities was approximately 2 $\mu$M under the present experimental conditions. Again, this represents the initial concentration of drug added to the reaction mixture prior to ultraviolet irradiation. The $I_{50}$ of the active form, the free radical, would be considerably lower than this value, but the precise figure cannot be estimated.

## DISCUSSION

This study was initiated to explore the nature of the interaction between chlorpromazine free radical and the $(Na^+ + K^+)$-ATPase system. It was demonstrated that the reaction between the chlorpromazine free radical and the enzyme protein proceeded very rapidly at pH 5.8. Because of the extremely rapid decay of the free radical, a study of decay kinetics was not possible at pH 7.5, where the enzymatic studies were carried out. However, since this enzyme preparation can be stimulated, although to a somewhat lesser degree, by the simultaneous presence of $Na^+$ and $K^+$ at pH 5.8 as well as at pH 7.5, one would expect the protein molecule to have essentially the same configuration at both pH values. Thus, it may be reasonable to assume that at pH 7.5, the reaction between chlorpromazine free radical and the enzyme protein proceeds rapidly, at a velocity not very much different from that observed at pH 5.8.

Treatment of the enzyme with 1.0 M NaCl restored the enzyme activity inhibited by chlorpromazine free radical, indicating that the inhibitor bound to the enzyme may be removed by exposure to a solution of high ionic strength or to $Na^+$. Studies by others (7) have shown that this enzyme system has a specific affinity for $Na^+$. Squires also reported (4) that the presence of NaCl or KCl prevented the progressive enhancement of inhibition when the enzyme was previously incubated in the presence of chlorpromazine. However, this phenomenon may have been due to the increased decay of chlorpromazine free radical at high ionic strength (17).

Cysteine and dithiothreitol also restored the enzyme activity inhibited by chlorpromazine free radical when added to the incubation mixture containing the inhibited enzyme preparation. Denaturation of the enzyme system by incubation with the chlorpromazine free radical in the absence of ATP is thus excluded, since the inhibited enzyme was reactivated by NaCl or sulfhydryl reagents. Oxidation of the enzyme protein, sensitized in the presence of chlorpromazine by ultraviolet light, is also excluded, since NaCl, but not reducing agents, such as sodium metabisulfite and ascorbic acid, reactivated the enzyme. This was also observed in our previous study (8), in

which chlorpromazine free radical generated by enzymatic or chemical procedures was found to be a potent inhibitor of the enzyme system.

Since inhibition of the $(Na^+ + K^+)$-ATPase system by chlorpromazine free radical would appear to be of the pseudo-irreversible or irreversible type as defined by Webb (20), kinetic analysis has many limitations. It is also complicated by the fact that at least two different enzyme systems, $(Na^+ + K^+)$-ATPase and $Mg^{2+}$-ATPase, are affected by the same inhibitor, and the analysis depends on the measurement of differences between the two inhibited velocities. This is particularly true when the $(Na^+ + K^+)$-ATPase velocity is reduced at low KCl concentrations. However, the present enzyme preparation, with high ATPase activity and less than 8.5% contamination with $Mg^{2+}$-ATPase activity under the standard assay conditions, gives good resolution of the $(Na^+ + K^+)$-ATPase activity.

The difficulty in the estimation of the chlorpromazine free radical at low steady state concentrations (8) makes it impossible to estimate kinetic constants. Even the $I_{50}$ calculated for chlorpromazine has limited significance, since its reliability depends upon such factors as duration and intensity of ultraviolet irradiation, enzyme concentration, and preliminary incubation time. Nevertheless, the enzyme preparation employed in the present study, a microsomal fraction treated with deoxycholate and NaI, appeared to be almost twice as sensitive to the inhibitory action of chlorpromazine free radical as the enzyme employed in the earlier study (8). Both studies were performed under identical experimental conditions, and therefore the $I_{50}$ values may be compared. The enzyme used in the present study has a higher specific $(Na^+ + K^+)$-ATPase activity and a lower $Mg^{2+}$-ATPase activity than the preparation used previously. Partial purification of the enzyme system by deoxycholate treatment of the microsomal fraction has been reported by Robinson *et al.* (6) to increase its sensitivity to chlorpromazine, although the involvement of the free radical form was not considered.

A conventional Lineweaver-Burk plot for interaction among $(Na^+ + K^+)$-ATPase, chlorpromazine free radical, and KCl was indicative of uncompetitive inhibition (19, 20). However, the distinction between uncompetitive and noncompetitive inhibition is rather difficult, since it depends on extrapolation of the regression lines, and the slope of the lines is determined by the points representing low velocity, with possible large errors. Furthermore, the "curves" seem to bend upward at low KCl concentrations, although linear regression lines were fitted to the plot.

The distinction between these two types of inhibition is easier with a Hunter-Downs plot (21), since the differentiation depends on the shape of the curve and does not require extrapolation of the data. A detailed kinetic interpretation may not be possible from the present data, since such an analysis can be made only with complete understanding of the nature of interaction among the inhibitor, the enzyme system, ATP, $Mg^{2+}$, $Na^+$, and $K^+$. However, analysis of the kinetic behavior of chlorpromazine free radical as an inhibitor of $(Na^+ + K^+)$-ATPase activity suggests that this molecule has actions similar to those of the organomercury compound *p*-hydroxymercuribenzoate, but unlike those of the cardiac glycoside ouabain. Inhibition of the $(Na^+ + K^+)$-ATPase activity by ouabain was enhanced when the KCl concentration in the incubation medium was low. In contrast, the inhibition of enzyme activity by chlorpromazine free radical or *p*-hydroxymercuribenzoate was reduced when the KCl concentration was low. Such results may be indicative of competitive and noncompetitive inhibition for ouabain and of uncompetitive inhibition for chlorpromazine free radical and *p*-hydroxymercuribenzoate (20).

A different interpretation is also possible. The ATPase reaction seems to involve at least two steps (7, 16).

$$E + \mathrm{ATP} \xrightarrow{Na^+} E - \mathrm{P} + \mathrm{ADP} \qquad (1)$$

$$E - \mathrm{P} \xrightarrow{K^+} E + \mathrm{P_i} \qquad (2)$$

One explanation of the reaction is that the

first step is $Na^+$-dependent while the second step is $K^+$-dependent (7). Ouabain seems to exert its inhibitory effect on the over-all ATPase reaction by inhibiting primarily the second step (16). Thus, at low $K^+$ concentrations, when the second step is rate-limiting, the inhibition of enzyme activity by ouabain is enhanced. If the effect of the chlorpromazine free radical is to inhibit the first step in the reaction, one would expect less inhibition at low $K^+$ concentrations. This result was observed. The fact that chlorpromazine free radical inhibits both over-all ATPase activity and $K^+$-activated *p*-nitrophenyl phosphatase activity to the same extent in the presence of 15 mM KCl may vitiate this hypothesis, particularly if the latter activity is a model for the second step of the ATPase reaction (16). Equally acceptable explanations are that both steps are sensitive to chlorpromazine free radical or that the over-all ATPase reaction proceeds in one step.

The observation that inhibition of the $(Na^+ + K^+)$-ATPase activity by chlorpromazine free radical is reduced at low KCl concentrations is in contrast to our previously published study (8) and that of Judah and Ahmed (3). However, the difference in enzyme preparation and/or the difference between the action of chlorpromazine free radical and chlorpromazine itself may be responsible for this discrepancy. The present enzyme preparation is more rigorously purified with a high concentration of NaI. $Na^+$ has been shown to affect the configuration of the enzyme protein (4, 22, 23).

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