# Inhibition of Brain Sodium- and Potassium-Stimulated Adenosine Triphosphatase Activity by Chlorpromazine Free Radical

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SUMMARY

Ultraviolet light was observed to have a profound effect on the inhibition *in vitro* of rat brain microsomal sodium- and potassium-stimulated ATPase activity [$Mg^{2+}$-dependent, ($Na^+ + K^+$)-stimulated ATP phosphohydrolase, EC. 3.6.1.3] by chlorpromazine.

The inhibition of the enzyme activity was minimal when the experiments were carefully performed, avoiding exposure to light. However, a significant inhibition of ($Na^+ + K^+$)-ATPase activity was observed when the chlorpromazine free radical was added to the enzyme mixture after it had been generated either by ultraviolet irradiation or by chemical oxidation (with sulfuric acid) of chlorpromazine solutions. The concentration required for 50% inhibition of enzyme activity ($I_{50}$) was 40 $\mu$M. Even greater inhibition was observed when the chlorpromazine free radical was generated by ultraviolet irradiation in a mixture containing enzyme. Under these experimental conditions, the $I_{50}$ was 3.5 $\mu$M. The ultraviolet irradiation of the drug-enzyme mixture failed to enhance the inhibition of ($Na^+ + K^+$)-ATPase activity when inhibitory concentrations of ouabain or *p*-hydroxymercuribenzoate were used. A positive correlation was observed between the amount of chlorpromazine transformed by ultraviolet irradiation and the enhancement of inhibition of ($Na^+ + K^+$)-ATPase activity by a corresponding amount of irradiation of the chlorpromazine-enzyme mixture. An enhanced inhibition ($I_{50}$ = 15 $\mu$M) was also observed when the chlorpromazine free radical was generated in the ATPase assay system in the simultaneous presence of peroxidase and hydrogen peroxide. The inhibition of ($Na^+ + K^+$)-ATPase activity by chlorpromazine sulfoxide was minimal even in the presence of peroxidase and hydrogen peroxide. It is concluded that a semiquinone free radical of chlorpromazine, rather than chlorpromazine itself, may be responsible for the inhibition of sodium- and potassium-stimulated ATPase activity *in vitro*.

## INTRODUCTION

Since 1957, when Skou (1) first described the presence of a $Mg^{2+}$-dependent ATPase activity (EC. 3.6.1.3) which is further stimulated by the simultaneous presence of $Na^+$ and $K^+$, substantial evidence has accumulated which indicates that this enzyme system is related to the active transport across cell membranes of cations (2), as well as of other substances (3–6). The enzyme system is specifically inhibited by low concentrations of cardiac glycosides (2). However, a variety of other agents are also capable of inhibiting this enzyme activity when they are present in high concentrations (2, 7–18). Among these agents is chlorpromazine, which has been shown also to inhibit the transport of several substances across membranes (19). Consequently, several papers have been published (10, 20–22) describing the inhibition of the ($Na^+ + K^+$)-stimulated ATPase system by

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chlorpromazine *in vitro*. In most laboratories where this agent has been studied, however, high concentrations of chlorpromazine have been required to inhibit this enzyme system *in vitro*.

During further studies of this phenomenon, it was observed that the exposure of chlorpromazine solution to light enhanced the inhibition of ATPase activity. When the chlorpromazine-enzyme mixture was exposed to sunlight or ultraviolet light, the inhibition was even greater, suggesting that a labile substance produced by the photoirradiation of chlorpromazine is involved. Since exposure of chlorpromazine solution to light is known to yield relatively unstable and highly reactive intermediary oxidation products, namely, semiquinone free radicals (23), the possibility that free radicals are responsible for the inhibition of $(Na^+ + K^+)$-ATPase activity *in vitro* has been explored.

## METHODS

White male rats, weighing 200–300 g, were killed by decapitation, and the brains were rapidly removed. After the meninges had been stripped away, two to three brains were homogenized in a Dounce ball-type homogenizer with 5 volumes of ice-cold solution containing 0.25 M sucrose, 5 mM histidine, 5 mM EDTA, and 0.2% deoxycholate, pH 6.8. The homogenate was centrifuged at 12,000 $\times g$ for 30 min. The supernatant was centrifuged at 35,000 $\times g$ for 30 min, and the pellet was suspended in a solution containing 0.25 M sucrose, 5 mM histidine, and 1 mM EDTA, pH 7.0. This suspension was centrifuged again at 35,000 $\times g$ for 30 min, and the pellet was resuspended in the above suspending solution. All the procedures were carried out at 2°. Protein was assayed by the method of Lowry *et al.* (24).

The ATPase activity of the microsomal fraction (0.016–0.032 mg of protein) was assayed at 37° in a total volume of 1.0 ml containing 50 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, and 5 mM Tris-ATP, with or without 100 mM NaCl and 15 mM KCl. The reaction was started by adding Tris-ATP after a 5-min incubation period and was terminated 10 min later by the addition of 1.0 ml of ice-cold 15% trichloracetic acid solution, unless otherwise specified. The mixture was centrifuged at 600 $\times g$ for 15 min, and the inorganic phosphate liberated from ATP was determined in a 1.0-ml aliquot of the supernatant solution by the method of Bonting *et al.* (25). The $Mg^{2+}$-dependent ATPase activity was subtracted from the total ATPase activity to calculate $(Na^+$ and $K^+)$-dependent ATPase activity. The results are presented as the activity ratio, which represents the ratio of the activity in the presence of the drug compared to the control activity of the same enzyme preparation.

A Mineralight lamp, model R-51 (primary emission wavelength, 253.7 m$\mu$), with its filter removed, was used as the ultraviolet light source. The samples were exposed at about 23–25° in quartz cuvettes with a 1.0-cm light path at a distance of 45 cm from the lamp, unless otherwise indicated.

The molar drug concentrations are those prevailing during measurement of enzyme activity. When the drug-enzyme mixture was exposed to ultraviolet light prior to estimation of enzyme activity, enzyme and drug were in a solution containing 100 mM Tris-HCl buffer, pH 7.5. After ultraviolet exposure, this solution was diluted 2-fold with a solution containing the necessary ions.

Incubations were performed in the dark. To facilitate pipetting of ATP solutions under minimal illumination, a repeating dispenser (Hamilton, model PB 600-10) with a 10-ml, gas-tight syringe (Hamilton) was used. The absorption spectra were recorded with a Shimadzu MPS 50L recording spectrophotometer. Horseradish peroxidase (type I, *Reinheitzahl* = 0.66) was purchased from Sigma Chemical Company. Chlorpromazine and chlorpromazine sulfoxide were kindly supplied by Dr. Harry Green of Smith Kline and French Laboratories. EDTA and deoxycholic acid were dissolved in Tris base (Sigma 7–9) solution.

A standard solution of the chlorpromazine free radical for spectrophotometric assay was prepared freshly by the oxidation

of chlorpromazine with ceric sulfate. An equimolar amount of ceric sulfate in 2.4 N sulfuric acid was added to chlorpromazine dissolved in 2.4 N sulfuric acid with rapid mixing to prevent overoxidation of chlorpromazine. The absorbance of the mixture was measured in a spectrophotometer immediately on preparation, since the product was unstable and decayed rapidly even at this pH.

## RESULTS

*Effect of ultraviolet irradiation on inhibition of ($Na^+ + K^+$)-ATPase activity by chlorpromazine.* Control ATPase activity in the presence of $Na^+$, $K^+$, and $Mg^{2+}$ was approximately 30 μmoles of inorganic phosphate released per milligram of protein in 10 min; 6 μmoles of inorganic phosphate were released per milligram of protein in 10 min with $Mg^{2+}$ alone. Although both the ($Na^+ + K^+$)-ATPase and $Mg^{2+}$-ATPase activities were sensitive to chlorpromazine or its derivatives, the $Mg^{2+}$-ATPase activity was much less affected than was the ($Na^+ + K^+$)-ATPase activity under all experimental conditions tested. Therefore, only the data for the ($Na^+$ and $K^+$)-stimulated portion of the ATPase activity are reported in the present paper.

As can be seen in Table 1, inhibition of the ($Na^+ + K^+$)-ATPase activity by 50 μM chlorpromazine was minimal when the experiment was carefully carried out in the dark. When the chlorpromazine solution alone was exposed to ultraviolet light for 4 min at a distance of 45 cm and subsequently added to an unexposed enzyme solution, however, the enzyme activity was inhibited by 26 ± 6.7% (mean of five experiments ± standard error). When the chlorpromazine-enzyme mixture was exposed and the enzyme activity was determined after the exposure, the inhibition was markedly enhanced (97 ± 0.9%). Exposure of enzyme solution to ultraviolet light without chlorpromazine resulted in somewhat reduced activity, although this loss of activity was not statistically significant. Moreover, the addition of unexposed or ultraviolet-exposed chlorpromazine to ultraviolet-exposed enzyme resulted in a mere addition of these inhibitory effects, and there was a distinct difference in inhibitory

TABLE 1
*Effect of ultraviolet irradiation on inhibition of ($Na^+ + K^+$)-ATPase activity by chlorpromazine*

| Reagents | Enzyme | Inhibition of ($Na^+ + K^+$)-ATPase activity[a] |
|---|---|---|
| | | % |
| None | Exposed[b] | 6 ± 2.7 |
| Chlorpromazine (50 μM) | | |
| Unexposed | Unexposed | 7 ± 2.3 |
| Unexposed | Exposed[b] | 21 ± 2.7[c] |
| Exposed[b] | Unexposed | 26 ± 6.7[c] |
| Exposed[b] | Exposed[b] | 33 ± 4.9[c] |
| Exposed simultaneously in a mixture[d] | | 97 ± 0.9[c] |
| Ouabain (0.3 μM) | | |
| Unexposed | Unexposed | 34 ± 5.2[c] |
| Exposed simultaneously in a mixture[d] | | 41 ± 6.9[c] |
| *p*-Hydroxymercuribenzoate (1 μM) | | |
| Unexposed | Unexposed | 32 ± 4.5[c] |
| Exposed simultaneously in a mixture[d] | | 48 ± 4.5[c] |

[a] Mean of five experiments ± standard error.

[b] The drug solutions or enzyme solutions were exposed to ultraviolet light for 4 min at a distance of 45 cm from the source before addition to incubation mixtures.

[c] Significantly different from control; $p < 0.01$.

[d] The drug-enzyme mixture was exposed to ultraviolet light for 4 min at a distance of 45 cm prior to ATPase assay.

potency between those experiments in which the chlorpromazine was exposed separately and those in which the enzyme and chlorpromazine were simultaneously exposed to light. Ultraviolet exposure of ouabain (0.3 $\mu$M) or sodium *p*-hydroxymercuribenzoate (1 $\mu$M) failed to enhance the inhibition induced by these agents. Both are inhibitory to rat brain $(Na^+ + K^+)$-ATPase activity in the concentrations studied (Table 1). When the ouabain- or *p*-hydroxymercuribenzoate-enzyme mixture was exposed to ultraviolet light, the total inhibition was comparable to the inhibition obtained with unexposed drug and light-exposed enzyme.

The dose-response curves (Fig. 1) indicate that when the chlorpromazine solution alone was exposed to ultraviolet light under standard conditions and was subsequently added to the enzyme solution (curve *B*), the inhibition of $(Na^+ + K^+)$-ATPase activity was slightly greater than that observed with unexposed chlorpromazine (curve *A*). However, when the chlorpromazine solution was exposed to ultraviolet light for 4 min at a shorter distance (4 cm), where ultraviolet radiation intensity is estimated to be 20 times stronger than that of the standard exposure (i.e., 45 cm), and subsequently was added to the enzyme solution, the resulting inhibition of $(Na^+ + K^+)$-ATPase activity was significantly enhanced. The $I_{50}$ under these conditions was 45 $\mu$M (curve *C*). Overexposure of the chlorpromazine solution resulted in a lower inhibition. In a different series of experiments, in which the chlorpromazine solution was exposed to ultraviolet light at a distance of 4 cm for 30 min and subsequently added to the enzyme mixture, the resulting inhibition was $59 + 7.0\%$ (mean of five experiments $\pm$ standard error) at a chlorpromazine concentration of 50 $\mu$M. This may be compared with $82 \pm 7.5\%$ inhibition when chlorpromazine solution was exposed for only 4 min under the same conditions.

The time interval between the termination of drug exposure and the addition of the exposed solution to the enzyme mixture had a significant effect on the ultimate inhibitory action of the drug. Generally, the greater the elapsed time, the less inhibitory was the drug. In most experiments, this time was controlled precisely. In certain experiments, however, this precision was not possible, with the result that the inhibitory effect of the same concentration of ultraviolet-exposed chlorpromazine differed slightly from one series of experiments to another. Experiments performed with the simultaneous ultraviolet exposure of drug and enzyme gave more consistent results, since the difference in elapsed time (see above) was not a factor. When the drug-enzyme mixture was exposed to ultraviolet light under standard conditions and enzyme

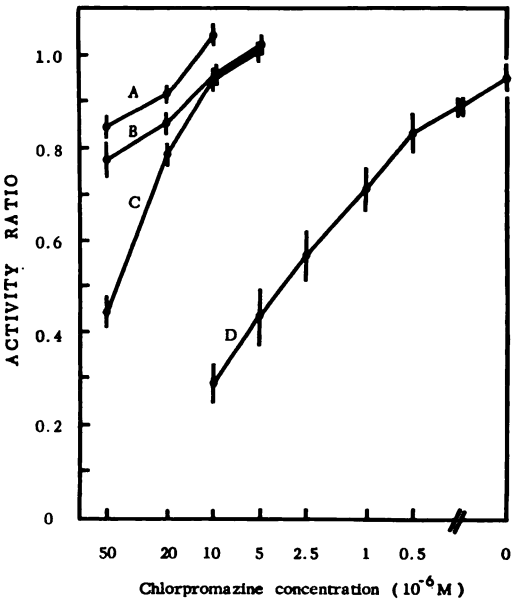


FIG. 1. *Inhibition of $(Na^+ + K^+)$-ATPase activity by chlorpromazine, and the effect of ultraviolet irradiation*

Unexposed chlorpromazine solution (*A*) or chlorpromazine solution exposed to ultraviolet light for 4 min at a distance of 45 cm or 4 cm from the source (*B* and *C*, respectively) was added to an incubation mixture containing enzyme; or chlorpromazine-enzyme mixture was exposed to ultraviolet light at a distance of 45 cm from the source for 4 min (*D*) before assay of ATPase activity. The incubation was carried out for 10 min at 37° after a 5-min preliminary incubation period. The abscissa indicates the final concentrations of chlorpromazine initially added to the incubation mixture. Values are the means of five experiments. Vertical lines indicate standard errors.

activity was determined after the exposure, the dose-response curve shifted markedly, the $I_{50}$ for chlorpromazine being approximately 3.5 $\mu$M (curve *D*).

Chlorpromazine solution normally has a sharp absorption peak at 255 m$\mu$. Ultraviolet irradiation of chlorpromazine dissolved in 50 mM Tris-HCl buffer, pH 7.5, resulted in a decrease in this absorption at 255 m$\mu$ and a corresponding increase at 240 m$\mu$, as can be seen in Fig. 2, which shows the absorption spectra of chlorpromazine solution before ultraviolet exposure (curve *A*) and those observed after 1, 2, 4, and 8 min of ultraviolet irradiation (curves *B*, *C*, *D*, and *E*, respectively). From the decreased absorbance at 255 m$\mu$ (curve *C*, 4-min exposure), it was estimated that approximately 35% of the chlorpromazine had disappeared during the standard exposure. The relationships between the amount of chlorpromazine transformed and the enhancement of the chlorpromazine inhibition of $(Na^+ + K^+)$-ATPase activity by ultraviolet light can be seen in Table 2, which summarizes the decrease in absorbance at 255 m$\mu$ due to ultraviolet irradiation of chlorpromazine solution and the effect of the same ultraviolet irradiation of the chlorpromazine-enzyme mixture on the $(Na^+ + K^+)$-ATPase activity. Chlorpromazine (10 $\mu$M) failed to inhibit $(Na^+ + K^+)$-ATPase activity unless the chlorpromazine-enzyme mixture was exposed to ultraviolet irradiation. The inhibition increased markedly, however, as the intensity of ultraviolet irradiation on the chlorpromazine-enzyme mixture was increased. There is a positive correlation ($r = 0.75$, $p < 0.001$) between the reduction of absorbance at 255 m$\mu$ and the enhancement of the inhibition of $(Na^+ + K^+)$-ATPase activity by a corresponding amount of ultraviolet irradiation of the chlorpromazine-enzyme mixture (Table 2), indicating that it is a chlorpromazine transformation product, rather than chlorpromazine itself, which inhibits the enzyme activity.

Unexposed chlorpromazine has no apparent absorption in the visible range, and no change was observed in the absorption spectrum in the visible range after exposure to ultraviolet light at neutral pH. In 16 N sulfuric acid, where the disproportionation of chlorpromazine free radical is almost negligible (26), however, ultraviolet irradiation of chlorpromazine caused a change in absorption spectra in both the ultraviolet and the visible range, resulting in an absorption spectrum (Fig. 3, curve *C*) similar to that of a free radical produced from chlorpromazine by ceric sulfate oxidation (Fig. 3, curve *A*). From the apparent molar extinction coefficient at 527 m$\mu$, it was estimated that 36% of the chlorpromazine originally present had been changed and accumulated as the free radical upon ultraviolet exposure. This calculation is based on the assumption that the molar extinction coefficient of chlorpromazine free radical is 10 $mM^{-1}$ $cm^{-1}$ at 527 m$\mu$, as determined from the absorbance of free radical produced from chlorpromazine by ceric sulfate oxidation (Fig. 3, curve *A*). In the

TABLE 2

*Effect of duration of ultraviolet irradiation on degradation of chlorpromazine and on chlorpromazine inhibition of $(Na^+ + K^+)$-ATPase activity*

The chlorpromazine-enzyme mixture was exposed to ultraviolet light at a distance of 45 cm from the source for various periods prior to the determination of absorbance at 255 m$\mu$ or ATPase assay. The final concentration of chlorpromazine in the incubation mixture was 10 $\mu$M.

| Duration of ultraviolet exposure | Reduction of absorbance at 255 m$\mu$ | Inhibition of $(Na^+ + K^+)$-ATPase activity[a] |
|---|---|---|
| *min* | % | % |
| 0[b] | 0 | 0 ± 3.9 |
| 1 | 14.4 | 20 ± 5.1 |
| 2 | 23.5 | 40 ± 3.5 |
| 4 | 33.9 | 74 ± 2.8 |
| 8 | 43.9 | 82 ± 2.4 |

[a] The activity was calculated from a comparison with the control activity of the same enzyme preparation exposed to ultraviolet light under the same conditions without the addition of chlorpromazine. These control activities were not significantly different from that of the unexposed enzyme preparation, except after 8 min of exposure. Results are the means of five experiments ± standard errors.

[b] The zero time values were obtained from preparations that had not been exposed to ultraviolet light.

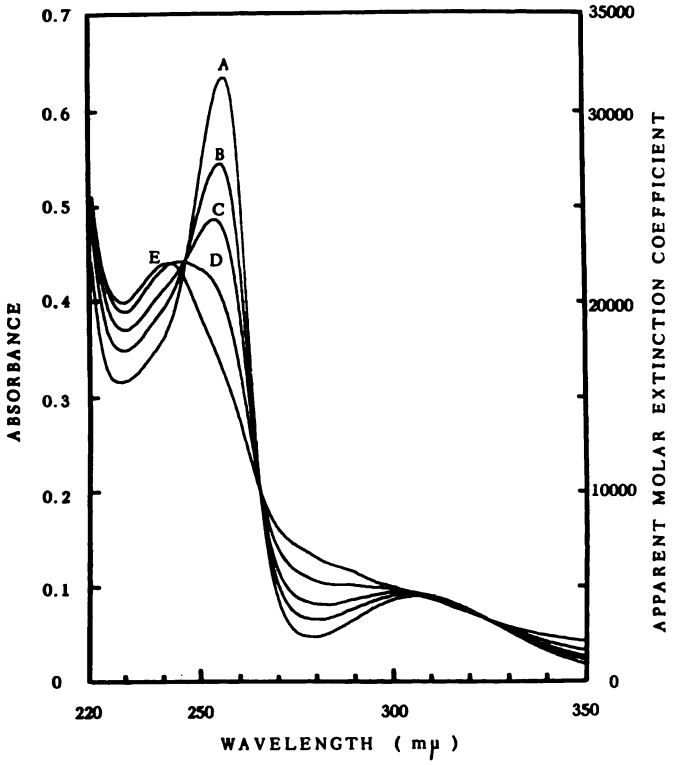


FIG. 2. *Changes in absorption spectra of chlorpromazine solution produced by ultraviolet irradiation*

A 20 μM chlorpromazine solution was prepared in 50 mM Tris-HCl buffer, pH 7.5. Absorption spectra were recorded immediately (*A*) and after exposure to ultraviolet light at a distance of 45 cm from the source for the duration of 1 min (*B*), 2 min (*C*), 4 min (*D*), and 8 min (*E*). The unit of molar extinction coefficient is liters per mole per centimeter.

latter oxidation, conversion of chlorpromazine to free radical is considered to have been almost complete (27).

*Effect of chemically produced chlorpromazine free radical on $(Na^+ + K^+)$-ATPase activity.* Chlorpromazine hydrochloride (17.75 mg) and chlorpromazine sulfoxide hydrochloride (18.55 mg) were dissolved together in 2.0 ml of 16 N sulfuric acid to produce the free radical according to the method of Levy and Burbridge (28). The solution immediately turned dark red, and 24 hr later, when the absorbance at 527 mμ became maximal, showed an absorption spectrum characteristic of chlorpromazine free radical (Fig. 3, curve *B*). From the absorbance at 527 mμ (Fig. 3, curve *B*), it was estimated that 78% of the sum of chlorpromazine and chlorpromazine sulfoxide was converted to the free radical.

This stock solution of chlorpromazine free radical and the corresponding sulfuric acid (used as the control) were adjusted to pH 3.0–3.5 by adding 0.5 M Tris base solution slowly under rapid mixing and then diluting 50-fold. Under these conditions, the free radical is fairly stable (26). Subsequent dilutions were made rapidly with distilled water. The solutions were added to the previously prepared, iced incubation mixture containing the ions, the microsomal preparation, and ATP. The reaction was started by transferring the test tubes to an incubation bath at 37°. The incubation was carried out immediately for 5 min. Figure 4, curve *A*, shows that a 40 μM concentration (expressed as the sum of chlorpromazine and chlorpromazine sulfoxide) produced 50% inhibition of $(Na^+ + K^+)$-ATPase activity. Control sulfuric acid had no

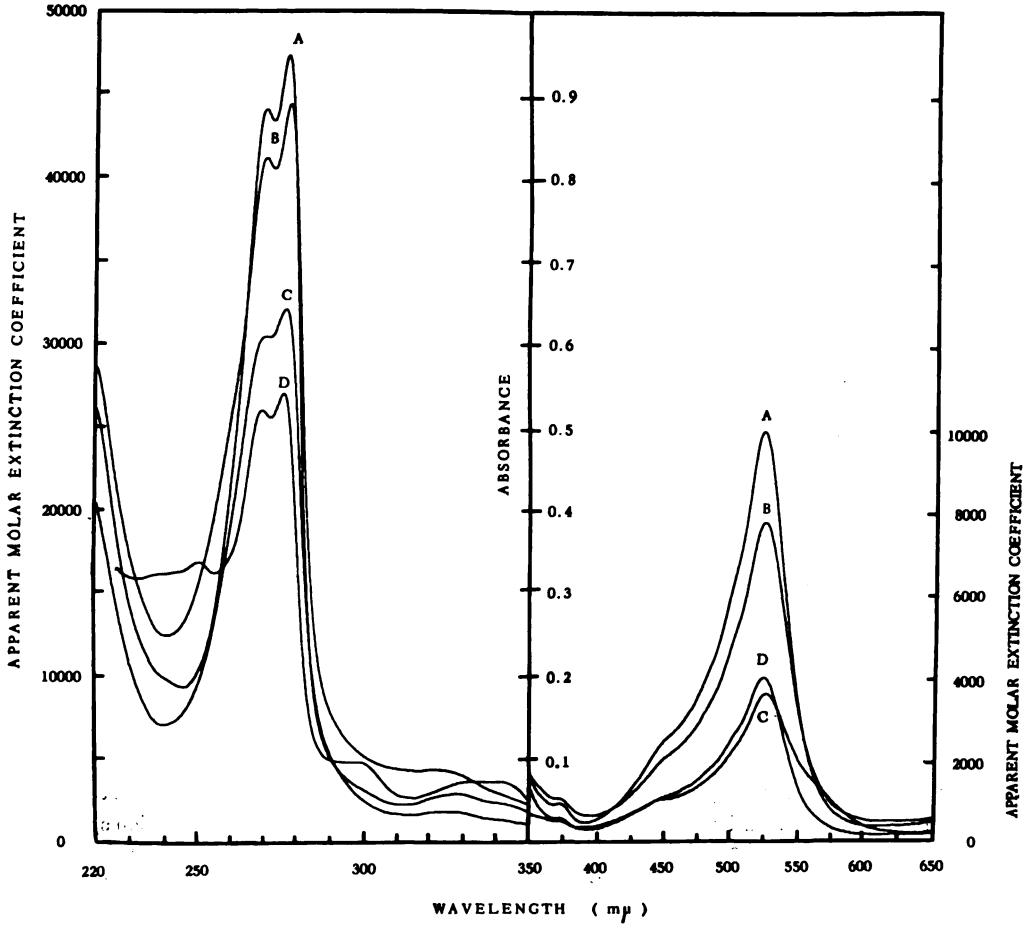


FIG. 3. *Absorption spectra of chlorpromazine free radicals produced by various methods*

Absorption spectra of 20 μM (for ultraviolet spectra) or 50 μM (for visible spectra) chlorpromazine were recorded after the following treatments: curve *A*, oxidation in 2.4 N sulfuric acid by an equimolar amount of ceric sulfate; *B*, equimolar amounts of chlorpromazine and chlorpromazine sulfoxide were dissolved in 16 N sulfuric acid and stored in the dark for 24 hr; the total concentrations of chlorpromazine and chlorpromazine sulfoxide initially added to the solution were 20 μM for the ultraviolet spectrum and 50 μM for the visible spectrum, respectively; *C*, chlorpromazine dissolved in 16 N sulfuric acid and exposed to ultraviolet light for 4 min at a distance of 45 cm from the source; *D*, chlorpromazine incubated with 0.4 μg of horseradish peroxidase per ml, 0.2 mM hydrogen peroxide, and 50 mM Tris-acetate buffer, pH 4.5, for 2 min at 25°.

effect on this enzyme system at the corresponding acid concentrations (Fig. 4, curve *B*), indicating that the inhibition observed with the free radical solution was not the result of acid addition. The pH did not change appreciably after the addition of the free radical solution which had been previously diluted and adjusted to pH 3.0. Nor was the enhanced inhibition caused by sulfate ion, although sulfate concentration was as high as 32 mM at the highest concentration employed, when the total concentration of chlorpromazine and chlorpromazine sulfoxide was 0.1 mM. Furthermore, when the free radical stock solution was adjusted to pH 7.0 and the free radical was allowed to decay before addition to the enzyme mixture, as evidenced by the complete disappearance of the red color, the inhibition of $(Na^+ + K^+)$-ATPase activity was negligible (Fig. 4, curve *C*) and not significantly different from that ob-

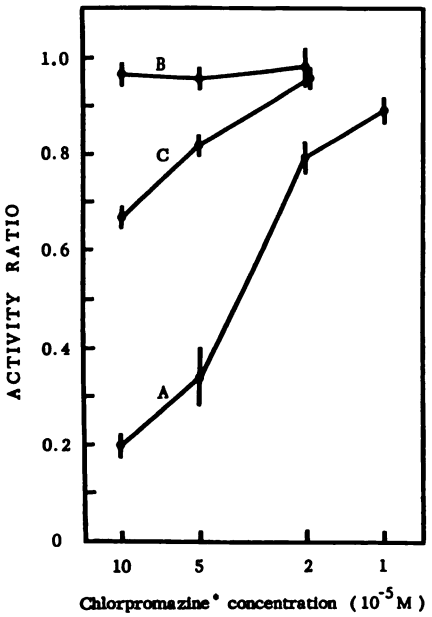


FIG. 4. *Effect of chlorpromazine free radical produced by sulfate treatment on $(Na^+ + K^+)$-ATPase activity*

Free radical was prepared by dissolving chlorpromazine hydrochloride (17.75 mg) and chlorpromazine sulfoxide hydrochloride (18.55 mg) in 2.0 ml of 16 N sulfuric acid. For curve *A*, free radical stock solution was adjusted to pH 3.0–3.5 by adding 0.5 M Tris base solution and then diluting 50-fold to make a 1 mM solution of each compound; subsequent dilutions were made rapidly with distilled water; *B*, 16 N sulfuric acid was treated likewise (acid control); *C*, free radical stock solution was adjusted to pH 7.0 by adding 0.5 M Tris base solution and diluting 50-fold. Ten minutes later, when the red color had disappeared completely, further dilutions were made with distilled water. The solutions were rapidly added to the previously prepared, iced incubation mixture containing the necessary cations, the microsomal preparation, and ATP. The incubation was carried out immediately at 37° for 5 min. Results are the means of five experiments. Vertical lines indicate standard errors. The concentrations recorded are the total levels of initially added chlorpromazine and chlorpromazine sulfoxide.

served with unexposed chlorpromazine (Fig. 1, curve *A*), again indicating that the free radical form of chlorpromazine reduces the $(Na^+ + K^+)$-ATPase activity.

*Effect of enzymatically induced chlorpromazine free radical on $(Na^+ + K^+)$-ATPase activity.* Incubation of chlorpromazine in 50 mM Tris-acetate buffer, pH 4.5, with 0.4 μg of horseradish peroxidase per ml and 0.2 mM hydrogen peroxide at 25° produced a red semiquinone free radical, as reported by Cavanaugh (29) and Piette *et al.* (30). The absorption spectrum of this mixture 2.0 min after the addition of chlorpromazine showed typical absorption peaks at 270, 277, and 527 mμ, indicating the generation of the free radical of chlorpromazine (Fig. 3, curve *D*). It also resulted in the formation of a small amount of chlorpromazine sulfoxide under these experimental conditions, since absorption peaks at 240, 300, and 340 mμ, characteristic of chlorpromazine sulfoxide (Fig. 5, curve *E*), were also observed in this spectrum (Fig. 3, curve *D*). The other peak of chlorpromazine sulfoxide at 275 mμ (Fig. 5, curve *E*) was not apparent, because it was masked by the large absorption peaks of the chlorpromazine free radical.

At pH 6.0, where the free radical is less stable, the development of a red color was not apparent. However, substantial amounts of chlorpromazine had disappeared during incubation at 37°. After a 15-min incubation at this temperature, chlorpromazine sulfoxide was found in the incubation mixture (Fig. 5, curve *C*). When 2.0 μg of peroxidase per ml of incubation mixture were added, the formation of chlorpromazine sulfoxide was more conspicuous (Fig. 5, curve *D*). In this case, the absorption spectrum was similar to that of the "decayed free radical" described by Borg and Cotzias (27), with an apparent absorption peak at 250 mμ. These results indicate that the peroxidase system was still oxidizing chlorpromazine at this pH and temperature. From the decrease of absorption at 255 mμ, it was estimated that 14% of the chlorpromazine had disappeared during the 15-min incubation period, a period comparable to the total preliminary and final incubation times for the ATPase assay.

The effect of the peroxidase-hydrogen peroxide system on chlorpromazine inhibition of $(Na^+ + K^+)$-ATPase activity at pH 6.0 can be seen in Fig. 6. The presence of 0.4 μg of horseradish peroxidase per ml and

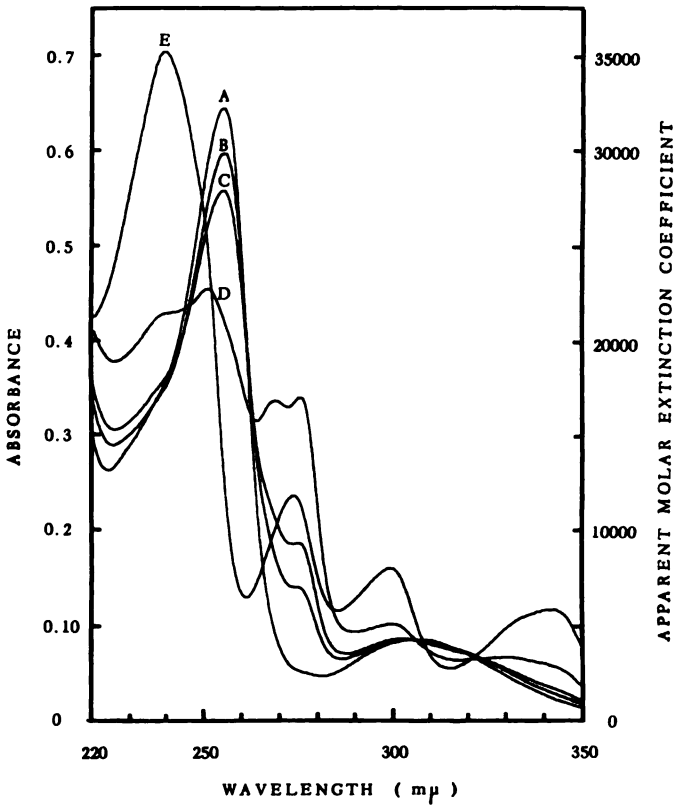


FIG. 5. *Changes in absorption spectra of chlorpromazine solution produced by a peroxidase-hydrogen peroxide system at pH 6.0 and 37°*

Absorption spectra of an incubation mixture containing 0.4 μg of horseradish peroxidase per ml, 0.2 mM hydrogen peroxide, and 50 mM Tris-HCl buffer, pH 6.0, were recorded before (*A*) and after 5 (*B*) and 15 (*C*) min of incubation at 37°. The reference cell contained corresponding concentrations of peroxidase and hydrogen peroxide. Curve *D* was obtained after a similar 15-min incubation with 2.0 μg of peroxidase per milliliter. Curve *E* is the absorption spectrum of 20 μM chlorpromazine sulfoxide solution.

0.2 mM hydrogen peroxide in themselves had no effect on $(Na^+ + K^+)$-ATPase activity (curve *A*). However, the inhibition of the enzyme activity by chlorpromazine was significantly greater in the presence of the peroxidase-hydrogen peroxide system when compared with the control experiments, in which chlorpromazine and hydrogen peroxide, but no peroxidase, were added (curve *B*). The $I_{50}$ for chlorpromazine under these experimental conditions (i.e., the peroxidase-hydrogen peroxide system) was 15 μM. Chlorpromazine sulfoxide, which does not yield a free radical on oxidation, did not inhibit $(Na^+ + K^+)$-ATPase activity under the same experimental conditions, even with a chlorpromazine sulfoxide concentration as high as 50 μM (curve *C*), indicating that it is the intermediary product formed along the oxidative pathway from chlorpromazine to chlorpromazine sulfoxide that is capable of inhibiting $(Na^+ + K^+)$-ATPase activity *in vitro*.

## DISCUSSION

The inhibition of $(Na^+ + K^+)$-ATPase activity was enhanced significantly when chlorpromazine solution was exposed to ultraviolet light before addition to the incubation mixture containing enzyme. The ultraviolet exposure of the drug-enzyme mixture prior to assay resulted in even greater enhancement of the inhibition, and under certain conditions it was possible to

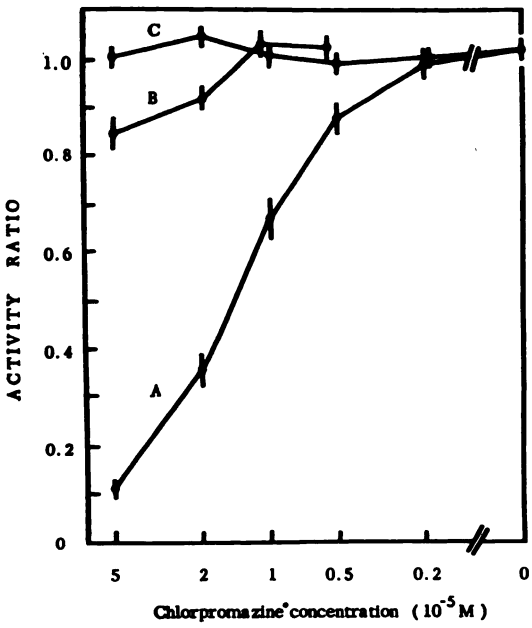


FIG. 6. *Inhibition of $(Na^+ + K^+)$-ATPase activity by chlorpromazine and chlorpromazine sulfoxide in the presence of a peroxidase-hydrogen peroxide system*

Chlorpromazine (*A*) or chlorpromazine sulfoxide (*C*) was added to the incubation mixture containing 0.4 μg of peroxidase per ml, 0.2 mM hydrogen peroxide, microsomal preparation (0.016 mg of protein), 50 mM Tris-HCl buffer (pH6.0), and the cations. The ATPase assay was carried out at 37° for 10 min after a 5-min preliminary incubation period. The control for chlorpromazine (*B*) was obtained by omitting peroxidase from the system containing chlorpromazine and hydrogen peroxide. Results are the means of five experiments. Vertical lines indicate standard errors. The concentrations indicated are those of chlorpromazine or chlorpromazine sulfoxide.

observe a 40-fold increase in chlorpromazine potency in inhibiting this enzyme system. When the chlorpromazine-enzyme mixture was exposed to various amounts of ultraviolet light, the amount of chlorpromazine transformed by ultraviolet irradiation was correlated with enhancement of the inhibition. Therefore, it seems reasonable to assume that a transformed species of chlorpromazine, but not chlorpromazine itself, is the active inhibitory molecule.

Although it is claimed that the enzyme $(Na^+ + K^+)$-ATPase is sensitive to ultraviolet irradiation (31), the enhancement of the inhibition seen in the present study is not the result of a change in the enzyme resulting from ultraviolet irradiation, since neither unexposed nor ultraviolet-irradiated chlorpromazine produced a greater effect on the ultraviolet-exposed enzyme than on the unexposed enzyme preparation. In the absence of chlorpromazine, the ATPase activity was only slightly affected by the standard conditions of ultraviolet exposure, indicating that such conditions are not strong enough to induce a significant change in the enzyme although they have a pronounced effect on chlorpromazine. Furthermore, photo-activation is unique with respect to chlorpromazine, because ultraviolet exposure did not enhance the actions of other $(Na^+ + K^+)$-ATPase inhibitors, ouabain and *p*-hydroxymercuribenzoate, on this enzyme system. The difference between the inhibitory potency observed when chlorpromazine was exposed alone before being added to the enzyme solution and that observed when chlorpromazine was exposed to ultraviolet light in a mixture containing enzyme indicates that the active species of chlorpromazine is unstable and short-lived.

Ultraviolet irradiation of chlorpromazine solution has been claimed to yield a semiquinone free radical (23) that is relatively stable yet does not exist very long at neutral pH (26). Semiquinone free radicals prepared from chlorpromazine by chemical, enzymatic, or electrochemical oxidation have a red color with absorption peaks at 270, 277, and 527 mμ (27–30, 32–34). The absorbance at 527 mμ was correlated with the intensity of the signal in electron spin resonance spectroscopy by these authors, and therefore is considered to be characteristic of the free radical. Borg and Cotzias (27) and Piette and Forrest (33) reported that the semiquinone free radicals prepared by these methods differ from the free radical prepared by photo-oxidation. This premise is based on the differences in the absorption spectra. These authors observed only a green, blue, or brown, but not a red, color on ultraviolet exposure of chlorpromazine solution. On the other hand, Felmeister and Discher (23) reported that

ultraviolet irradiation is capable of producing a red semiquinone free radical when the exposure of the chlorpromazine solution is carried out in 9 N sulfuric acid. The present data confirm the report of Felmeister and Discher (23). At neutral pH, however, ultraviolet exposure of chlorpromazine solution did not produce a red color or an ultraviolet absorption spectrum characteristic of the free radical. The only changes observed under our standard conditions of ultraviolet irradiation were a decrease in absorption at 255 mμ, indicating the disappearance of chlorpromazine, and an increase in absorption at 240 mμ. Extremely intense ultraviolet irradiation resulted in the green, blue, and finally brown color described by Borg and Cotzias (27) and Piette and Forrest (33), without producing a red color even during the early stages of irradiation. The failure of these authors to observe a red free radical upon ultraviolet exposure probably resulted from the rather intense ultraviolet irradiation they used, as well as from the high pH of the solvent during exposure. Thus, the apparent differences observed by various investigators seem reconcilable, and we conclude that there are no essential differences between the free radicals prepared from chlorpromazine by different methods. The fact that ultraviolet irradiation produced a red product only in strong sulfuric acid was probably due to the rapid decay of the chlorpromazine free radical at neutral pH (26), especially during ultraviolet irradiation. Although ultraviolet irradiation enhances the degradation of chlorpromazine and chlorpromazine sulfoxide, it likewise seems to facilitate the decay of free radical, because strong ultraviolet irradiation is capable of bleaching out the red color of a free radical solution prepared by various methods. Unless the decay is reasonably slow, the free radical does not accumulate to a concentration sufficiently high to be detected spectrophotometrically. From the molar extinction coefficient of 10 $mM^{-1}$ $cm^{-1}$ at 527 mμ and the rather wide absorption peak, it is estimated that at least 1 μM of chlorpromazine free radical would be required in order to be detected at visible wavelengths. The concentration should be even higher if the molar extinction coefficient is 7.8 $mM^{-1}$ $cm^{-1}$, as reported by Borg and Cotzias (27). The differences in molar extinction coefficient values might be due to the fact that the ceric sulfate oxidation and determination of absorbance were carried out at a lower pH in the present investigation.

A possible pathway for the oxidation of chlorpromazine to chlorpromazine sulfoxide, which involves the generation and decay of the chlorpromazine free radical, is shown in Fig. 7. Both the free radical and the phenazathionium ion would be produced by mechanisms capable of effecting a 1-electron oxidation of chlorpromazine. The premise that a free radical may be the active species for the inhibition of $(Na^+ + K^+)$-ATPase activity is based on the following facts: (a) free radicals are highly reactive with protein, and (b) the phenazathionium ion is extremely unstable and readily reacts with water rather than protein to yield chlorpromazine sulfoxide. Therefore it would appear likely that a free radical is the active species.

The steady-state concentration, which is

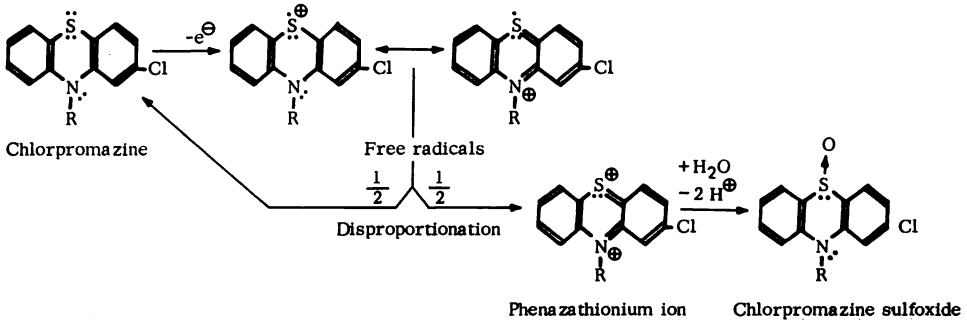


FIG. 7. *The generation and disproportionation of chlorpromazine free radicals*

the function of the total amount of free radical produced in a given period of time and its half-life, determines the rate of interaction between the free radical and the enzyme molecule. Although a steady-state concentration of the free radical could not be determined experimentally at neutral pH values, it may be estimated to be lower than 1 $\mu$M, since no red color was observed under these experimental conditions during ATPase assay. The amount of chlorpromazine transformed was 1 $\mu$M as observed by ultraviolet irradiation, or 2 $\mu$M with the peroxidase-hydrogen peroxide system, when the $(Na^+ + K^+)$-ATPase activity was inhibited by 50%. From the extremely rapid decay of free radical at neutral pH (26), it is presumed that the steady-state concentration of the free radical is certainly well below these concentrations.

With respect to previous studies on the effect of chlorpromazine on $(Na^+ + K^+)$-ATPase, it is not possible to estimate the extent of participation of a free radical, because conditions of laboratory illumination are not described. The involvement of free radical seems to have been minimal in the experiments reported by Järnefelt (10), Judah and Ahmed (20), and from this laboratory (22), where 0.1 mM chlorpromazine inhibited less than 40% of the enzyme activity.

In contrast to Squires' results (21), preliminary studies indicate that the presence of $Na^+$ or $K^+$ during ultraviolet exposure of a chlorpromazine-enzyme mixture does not reduce the inhibition if these ion concentrations are kept constant during the ATPase assay. Furthermore, a low $K^+$ concentration during the ATPase assay results in a greater inhibition by chlorpromazine, an observation in accordance with the work of Judah and Ahmed (20).

Guth and Spirtes (19) warned that since chlorpromazine has a profound effect on membrane permeability in general, any change observed with chlorpromazine might be a secondary phenomenon, i.e., the result of a change in the accessibility of the substrate to the enzyme. The present microsomal preparation, however, was treated with relatively high concentrations of deoxycholate during enzyme preparation, a process likely to impair the membrane structure. Thus, it is unlikely that the accessiblity of ATP could be a factor limiting enzyme activity. Two other facts should be noted. One is the high ATPase activity of our preparation, which would indicate an increased accessibility of substrate to enzyme, and the second is the use of high concentrations of ATP in the reaction medium, which would tend to reduce the effect of a membrane barrier. Thus it is less likely that the inhibition of the ATPase activity is secondary to a change in membrane permeability under our experimental conditions.

Levy and Burbridge (28) also demonstrated that a free radical formed from chlorpromazine is responsible for the inhibition *in vitro* of uridine diphosphate glucose dehydrogenase, an enzyme which does not contain any membrane structure. The concentration reported to inhibit this enzyme seems to be in the same range as required to inhibit $(Na^+ + K^+)$-ATPase activity. Thus it appears likely that the free radical of chlorpromazine is capable of inhibiting several different enzyme systems.

The major metabolite of chlorpromazine *in vivo* is known to be chlorpromazine sulfoxide (35), and it is probable that a free radical is produced during this biotransformation (Fig. 7). In the physiological pH range, however, the distance between the site of generation of a free radical and the site of its action on a biological system would be the factor determining whether the free radical is actually the pharmacologically active form. If the free radical is generated a short distance from the enzyme or is generated in a more acidic medium, and if $(Na^+ + K^+)$-ATPase is the target protein, the chlorpromazine free radical could be responsible for the effect of chlorpromazine *in vivo*.

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