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Stable Enzyme Inhibitors and Stable Free Radical Species in Ultraviolet-Irradiated Solutions of Chlorpromazine

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SUMMARY

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Irradiated solutions of chlorpromazine inhibited the activities of yeast alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1), beef heart lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27), glyceraldehyde phosphate dehydrogenase [D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12], and uridine diphosphoglucose dehydrogenase (uridine diphosphate glucose:NAD^+ oxidoreductase, EC 1.1.1.22) even after long periods of storage in darkness at 40°. Two classes of products were obtained from irradiated chlorpromazine. Solutions of these products both inhibited enzyme activities and contained relatively stable free radicals. During and following free radical decay, the inhibitory activity of the solutions containing them was essentially unchanged. It is concluded that stable photolytic products in these solutions, other than free radicals, contributed predominantly to the inhibition of enzyme activity observed.

INTRODUCTION

The suggestion has been made that a free radical formed from chlorpromazine may contribute to its psychopharmacological activity (1–4). The inhibition of enzymes by CPZ[1] *in vitro* has also been attributed to a free radical form of the molecule. From the results of enzyme inhibition studies, Akera, Brody and their colleagues (5–8) concluded that there are no important differences between the free radicals formed by ultraviolet irradiation of aqueous solutions of CPZ and those present in nearly neutral solutions of the solid free radicals prepared by chemical oxidation of CPZ. This assumption was also made explicitly by Wollemann and co-workers (9–11) in studies of the inhibition of D-glyceraldehyde 3-phosphate dehydrogenase and yeast alcohol dehydrogenase and the oxidation of NADH, and is implicit in the formulation by Levy and Burbridge (12) of the mechanism of inhibition of uridine diphosphate glucose dehydrogenase by irradiated CPZ.

The inhibitory activity was attributed by these investigators to the semiquinone free radical formed by oxidation of CPZ (5, 11, 12). This radical has been isolated as a red solid by Merkle, Discher, and Felmeister (13). Its ultraviolet spectrum was measured by Merkle and Discher (14), and its electron paramagnetic resonance spectrum was obtained by Levy and co-workers (4) and by Piette and Forrest (15). The radical is stable in concentrated strong

[1] The abbreviation used is: CPZ, chlorpromazine.

acids, but decays rapidly by second-order kinetics in solutions with low hydrogen ion concentration (16). At a concentration of 0.1 mM in nearly neutral solution, the half-life of this free radical is approximately 4 sec (17).

In a study of the inactivation of enzymes by irradiated solutions of CPZ, we have observed that the products that inhibit enzymes persist for relatively long periods in the dark in neutral solution. Furthermore, the degree of enzyme inhibition is proportional to the duration of exposure to inhibitor after irradiation, even when exposure times are as long as 0.5 hr. This observation suggests that these enzyme inhibitors are relatively stable entities rather than the short-lived free radicals produced by chemical oxidation. In this paper we show that irradiation of aqueous solutions of CPZ yielded at least two classes of products, one of which could be extracted into heptane and the other recovered from the heptane-extracted aqueous phase. Solutions of these products inhibited enzyme activities and contained relatively stable free radicals. During and following free radical decay, the inhibitory activity of the solutions containing them was essentially unaltered.

METHODS

The molarity of irradiated CPZ solutions will be given as that of the CPZ solutions exposed to irradiation. All operations were carried out in subdued light and in actinic glass containers.

One hundred-milliliter portions of a 0.15 mM solution of CPZ (Smith Kline & French) in 0.01 M phosphate buffer, pH 7.4, were placed in an open Pyrex container with an internal diameter of 11.0 cm. Radiation, predominantly at 253.7 nm, was provided by two Westinghouse germicidal lamps placed 11.0 cm above the CPZ solution. During irradiation the solution was maintained at 3–5° with rapid stirring. After irradiation for 3 min, the absorbance at 255 nm was reduced by 30–40% of its original value. After irradiation for 4 min, it appeared that all of the CPZ present had been converted to photolytic products.

After the irradiated solution of CPZ had been held in a separatory funnel at 40° for 15 min, 15 ml of heptane were added, and the mixture was shaken and held for an additional 5–10 min at 40°. The resulting blue heptane phase was separated, and the extraction procedure was repeated a second time. The heptane extracts were combined and centrifuged, and the supernatant was flash-evaporated at 40°. Care was taken to exclude visible traces of water from the solution to be flash-evaporated. The heptane extract yielded a blue-green residue that dissolved readily in heptane, and less readily in phosphate buffer at pH 7.4.

The heptane-extracted aqueous portion was adjusted to pH 7 with 1 N H_2SO_4 and flash-evaporated at 40°. Within 1 hr a pink residue was obtained that dissolved readily in water or buffer, but not in heptane. A pink residue with the same EPR and absorption spectra was also obtained from solutions of CPZ irradiated at pH 4 and adjusted to pH 7 before flash evaporation. Solutions of CPZ irradiated at pH 4 did not yield a blue-green heptane phase when extracted at pH 7.4.

EPR spectra were obtained from solutions of the residue in 0.01 or 0.08 M phosphate buffer at pH 7.4 or other solvents. Samples were usually placed in 50-μl capillary tubes and measured at 25° by means of a Varian E3 EPR spectrometer. Absorption spectra and changes of absorbance at 340 nm with time were obtained with a Beckman DK 2A ratio-recording spectrophotometer. Solutions were analyzed in quartz cuvettes with a 1.0-cm light path at 37°. The radioactivity of aqueous and heptane solutions of ^{35}S-containing samples mixed with Amersham/Searle "PCS" solubilizer was determined with a 720 series Nuclear-Chicago liquid scintillation counter. The effects of irradiated solutions of CPZ and of solutions of the blue-green and pink residues on the activities of several enzymes were determined by standard assays of enzyme activity after incubation of the enzymes with these solutions.

RESULTS

Absorption spectra. Figure 1 shows that irradiation of CPZ produced a shift in the absorption maximum of the solution from

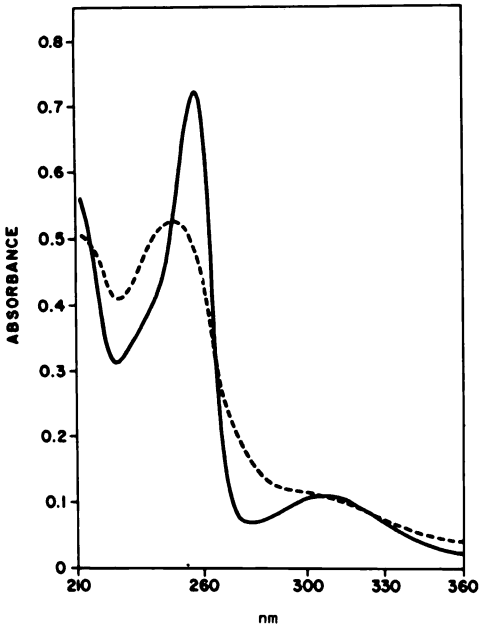

FIG. 1. *Absorption spectra of CPZ and irradiated CPZ at pH 7.5*

——, absorbance of 0.025 mM CPZ before irradiation; - - - after irradiation. CPZ was irradiated at pH 7.5.

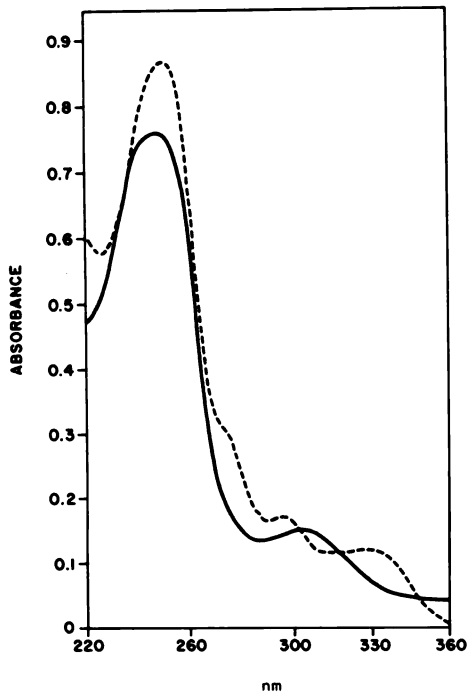

FIG. 2. *Ultraviolet absorption spectra at pH 6.0 of solutions of residues from irradiated CPZ*

——, absorbance of the blue-green residue; - - -, pink residue. The blue-green residue was obtained from heptane extraction of 300 ml of 0.15 mM CPZ irradiated at pH 7.5. The pink residue was obtained from 100 ml of 0.15 mM CPZ irradiated at pH 4.0. To measure ultraviolet absorbance, 150 ml and 300 ml of 0.048 M succinate buffer at pH 6.0 were added to the blue-green and the pink residues, respectively. Succinate buffer was the optical blank.

255 nm to 248 nm. Solutions of the blue-green and the pink residues retained this 248 nm absorption peak (Fig. 2) and exhibited similar ultraviolet spectra, but differed considerably at longer wavelengths, where the absorption maxima at pH 6 were 670 nm and 505 nm, respectively (Fig. 3).

The spectra of CPZ and of CPZ irradiated at pH 7.4, when dissolved in 16 N H_2SO_4, are shown in Fig. 4. The spectra of CPZ, which have been attributed to the semiquinone free radical, differed from

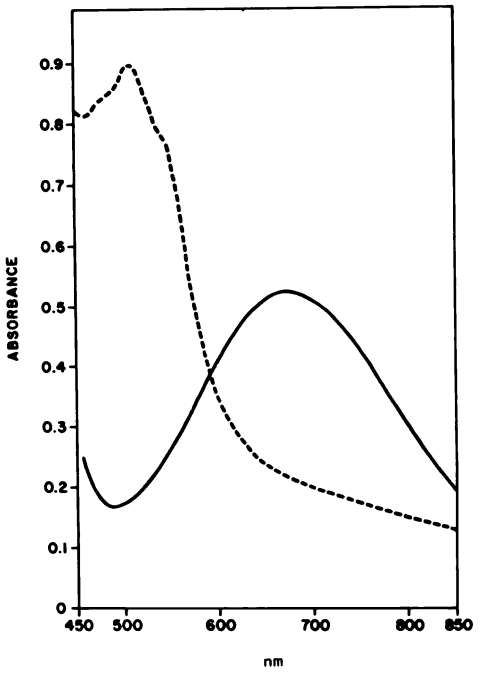

FIG. 3. *Visible absorption spectra at pH 6.0 of residues from irradiated CPZ*

Blue-green (——) and pink (- - -) residues were obtained as described in the legend to Fig. 2. To measure visible absorbance, 6.0 ml and 3.0 ml of 0.048 M sodium succinate at pH y were added to the blue-green and the pink residues, respectively.

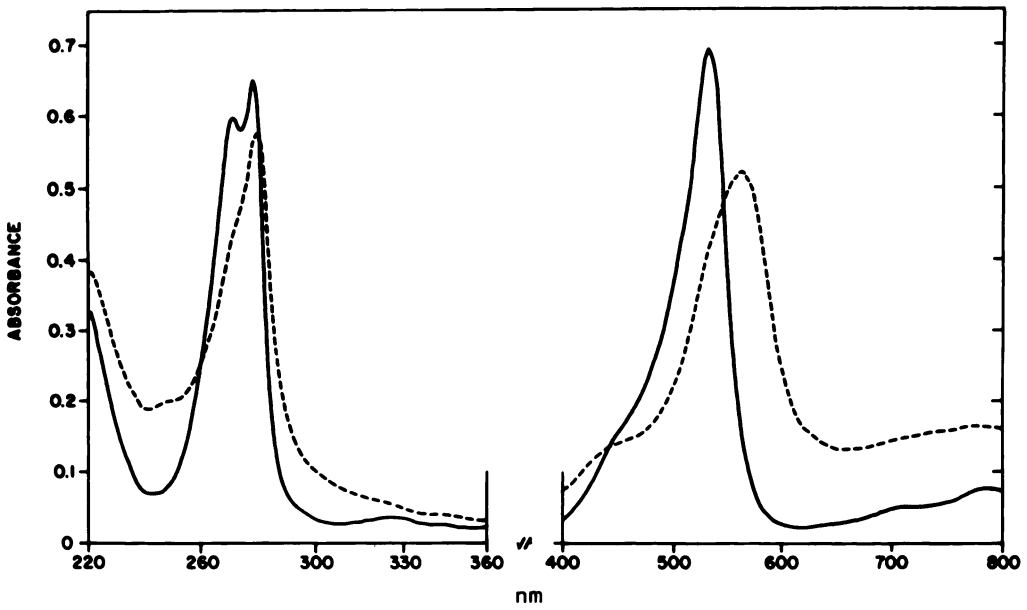

FIG. 4. *Visible and ultraviolet absorption spectra of CPZ and irradiated CPZ in 16 N H_2SO_4*
——, absorbance of CPZ before irradiation; - - -, after irradiation. Ultraviolet absorbance measurements were made on 0.015 mM CPZ, and visible absorbance measurements were made on 0.075 mM CPZ. The solvent was 16 N H_2SO_4.

those of irradiated solutions of CPZ. The bathochromic shift and symmetry of spectra of irradiated CPZ suggest that a large proportion of the CPZ in irradiated solutions was changed to precursors that yielded a purple solution in this acid, with an absorption maximum at 560 nm.

The absorption spectra of the blue-green and the pink residues dissolved in 16 N H_2SO_4 are shown in Fig. 5. The irradiation products in this solvent absorbed appreciably in the short-wavelength infrared region. The hydrophilic pink residue yielded a purple solution, and the less hydrophilic blue-green residue yielded a pink solution. The spectrum of the blue-green residue resembled that of the semiquinone free radical, but the ultraviolet absorption spectrum of this residue lacked the prominent double peak at 270 nm typical of the semiquinone.

EPR spectra of fractions from irradiated CPZ. Addition of phosphate buffer, pH 7.4, to the blue-green residue resulted in a solution containing a free radical, the EPR spectrum of which is shown in Fig. 6. Heptane solutions of this residue did not give an EPR signal. Figure 7 shows the EPR spectrum of an aqueous solution of the pink residue, obtained by vacuum-drying the aqueous phase of the heptane-extracted irradiated CPZ solution or by vacuum-drying CPZ solutions irradiated at pH 4. At pH 7.4 the EPR spectrum of the pink residue persisted for several days, and was essentially unchanged except that the strength of the signal diminished. The spectrum of the blue-green residue increased in intensity and, after about 1 hr, exhibited additional hyperfine structure (Fig. 8). After standing for about 1 day, this signal slowly decayed to a single line similar to but not identical with the spectrum of the pink residue. None of these spectra resembled that of the semiquinone free radical.

The EPR signals of aqueous solutions of both residues were quenched when the solutions were acidified to pH 4 with acetic acid. Adjustment of the acidified solutions to pH 7.4 restored the signals, but the signals were now diminished slightly in intensity and showed small changes of hyperfine structure.

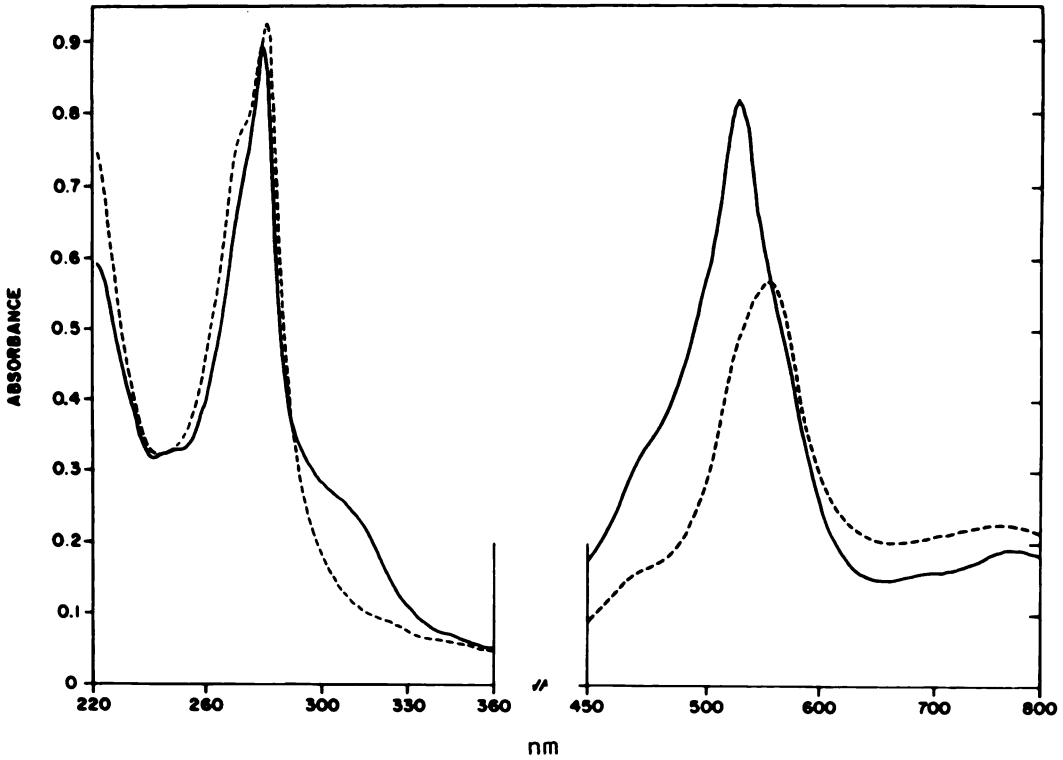

FIG. 5. *Ultraviolet and visible absorption spectra of blue-green and pink residues in 16 N H_2SO_4*
——, absorbance of the blue-green residue; – – –, pink residue. The residues were obtained as described in the legend to Fig. 2. For visible spectra, the blue-green residue was dissolved in 33 ml and the pink residue in 143 ml of 16 N H_2SO_4. For the ultraviolet spectra, the blue-green residue was dissolved in 165 ml and the pink residue in 550 ml of this acid.

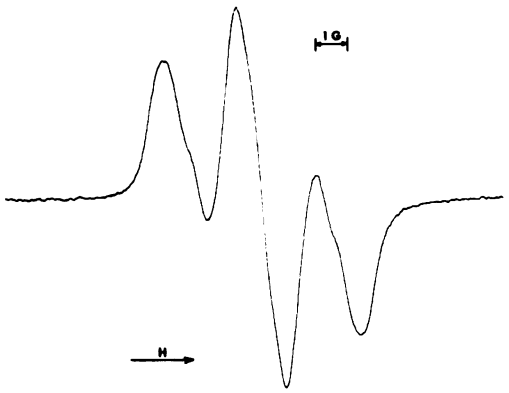

FIG. 6. *EPR spectrum of solution of blue-green residue at pH 7.4*

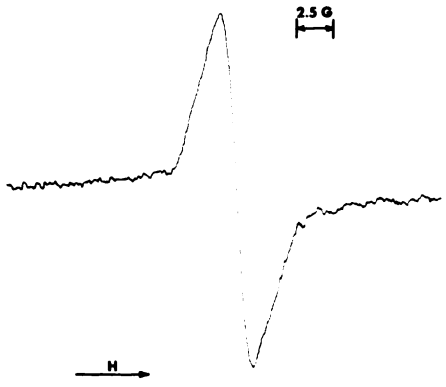

FIG. 7. *EPR spectrum of solution of pink residue at pH 7.4*

Distribution of photolytic products in aqueous and organic phases. To measure the partition of the photolytic products of CPZ between the aqueous and heptane fractions, solutions of [^{35}S]CPZ were irradiated and then fractionated with heptane. The data in Table 1 reveal that 99.7% of unirradiated CPZ was extracted into hep-

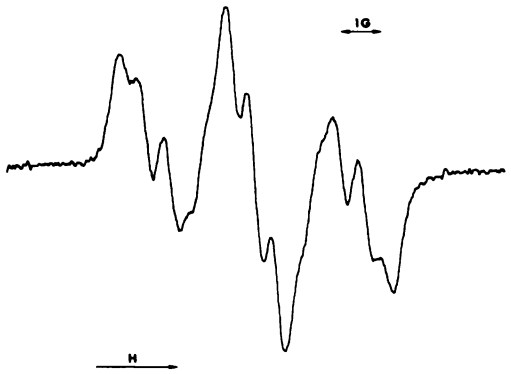

FIG. 8. *EPR spectrum of blue-green residue at pH 7.4 after having been in solution for 1 hr*

TABLE 1

Effect of irradiating [^{35}S]CPZ on distribution of isotope in pH 7.4 buffer–heptane system, and recovery of ^{35}S in heptane solution of blue-green residue

One hundred milliliters of a solution of unirradiated or irradiated [^{35}S]CPZ, containing respectively 1.29×10^7 or 7.1×10^6 cpm, were extracted two times with 10-ml portions of heptane. Radioactivity was determined in the pH 7.4, 0.015 M phosphate buffer and heptane phases, and in a heptane solution of the blue-green residue obtained from flash evaporation of 20 ml of the heptane extract of irradiated CPZ.

[^{35}S]CPZ	Buffer		Heptane		Residue	
	cpm $\times 10^{-6}$	%	cpm $\times 10^{-6}$	%	cpm $\times 10^{-6}$	%
Unirradiated	0.04	0.29	13.0	99.7		
Irradiated	4.1	57.0	3.0	43.0		
Irradiated	4.1	57.0			1.5	21.0

tane from a pH 7.4 buffer solution. Following irradiation and heptane extraction, 57% of the radioactivity remained in the aqueous phase. After the heptane fraction had been evaporated and the blue-green residue redissolved in heptane, its radioactivity was only 21% of the initial value. The 22% of the radioactivity not accounted for was probably in a brown scum that formed at the heptane-water interface and adhered to the walls of the separatory funnel.

Enzyme inhibition studies. The inhibition of crystalline yeast alcohol dehydrogenase (alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1) (Sigma Chemical Company) by irradiated solutions of CPZ was studied. Irradiated solutions of CPZ were brought to ice-bath temperature and diluted to 0.15 mM, 0.075 mM, and 0.015 mM CPZ with cold buffer to give a final volume of 0.95 ml. Solutions of unirradiated CPZ and the buffer alone served as controls. Portions (0.05 ml) of a solution of the enzyme, containing 0.28 mg of enzyme per milliliter, were incubated with the irradiated CPZ solutions for various periods of time at selected temperatures, after which 5 ml of cold buffered gelatin were added. The activity of the enzyme was then assayed in a system containing 1.0 ml of 0.03 M sodium pyrophosphate (pH 8.8), 1.5 ml of H_2O, 0.2 ml of 0.012 M NAD$^+$ and 0.2 ml of 95% ethanol. The reaction was started by the adddition to this system of 0.1 ml of the cold enzyme-inhibitor solution; enzyme activity was measured in terms of the rate of reduction of NAD$^+$ during incubation at 37°.

The results of this study are shown in Table 2. Irradiated CPZ, 0.015 mM, had no effect on enzyme activity after incubation at 36° for 1 min, but after incubation for 1.5 and 2 min enzyme activity was reduced by 40% and 90%, respectively. Incubation with 0.14 mM irradiated CPZ at 36° for only 1 min produced complete inhibition, but this concentration of irradiated CPZ produced only 72% inhibition after incubation for 10 min at 2°. By contrast, 0.14 mM CPZ produced only 25% inhibition after incubation at 36° for 2 min, and did not inhibit the activity of the enzyme when the incubation was carried out for 2 min at 2°.

The results of the study of the effects of irradiated solutions of CPZ on the activity of beef heart lactate dehydrogenase (L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) (Sigma) are shown in Tables 3 and 4. Solutions containing 0.15 mM CPZ were maintained in the dark at pH 7.4 and 4.0 and at 2° and 40° for periods up to 5760 min. At intervals 1.0-ml portions were added to 0.9 ml of buffer solution and brought to 37°. A 0.1-ml portion of a solution of lactate dehydrogenase (6.99 mg/ml) was added to each portion of the inhibitor solution, and the

TABLE 2

Activity of yeast alcohol dehydrogenase in CPZ and irradiated CPZ solutions at different concentrations and temperatures for various periods

Incubation was initiated by adding 0.05 ml (0.014 mg) of enzyme to 0.95 ml of buffer, CPZ, or irradiated CPZ. Incubation was terminated by adding 5.0 ml of cold buffered gelatin to each solution. Enzyme activity was determined by measuring the rate of NAD reduction at 340 nm after the addition of 0.1 ml of the cold solutions to 2.0 ml of assay system at 37°. Rate = change in absorbance per minute/6.22 × milligrams of enzyme per milliliter of reaction mixture. The enzyme was added to a solution made up of 1.0 ml of 0.03 M sodium pyrophosphate (pH 8.8), 1.5 ml of H_2O, 0.2 ml of 0.012 M NAD, and 0.2 ml of 95% ethanol. The enzyme was Sigma lot 1288-6180.

Enzyme incubated with	CPZ	Temperature	Time	Rate
	M × 10^5		*min*	
Buffer		2°	2	308
Buffer		36°	2	227
Unirradiated CPZ	14	2°	2	320
Unirradiated CPZ	14	36°	2	170
Irradiated CPZ	1.5	36°	2	24
Irradiated CPZ	1.5	36°	1.5	136
Irradiated CPZ	1.5	36°	1	227
Irradiated CPZ	3.0	36°	1	82
Irradiated CPZ	4.5	36°	1	75
Irradiated CPZ	14	36°	1	0
Irradiated CPZ	14	2°	10	53

mixture was incubated for 3 min, after which 4 ml of cold buffer were added. The assay was initiated by adding 0.1 ml of the enzyme-inhibitor solution to 2 ml of a reaction mixture containing 0.33 mM sodium pyruvate, 0.067 mM NADH, and 0.027 M phosphate buffer, pH 7.4. Enzyme activity was measured in terms of the rate of oxidation of NADH. In this study lactate dehydrogenase activity was not inhibited by incubation in 0.15 mM CPZ for 3 min at 37°, but 0.075 mM irradiated CPZ produced 69% inhibition of the enzyme. Table 3 shows that the inhibitory action of irradiated CPZ solutions at pH 7.4 depended strongly on the temperature at which the solutions were held, whereas the dependence on temperature was less pronounced when the solutions had been held at pH 4 (Table 4). The inhibitory activity of irradiated CPZ solutions maintained at pH 4

TABLE 3

Changes in inhibitory activity toward lactate dehydrogenase of irradiated CPZ solutions maintained at pH 7.4 at 2° and 40°

After 1-ml samples of 0.15 M irradiated CPZ had been maintained at the indicated times and temperatures, 0.9 ml of 0.15 M phosphate buffer at pH 7.4 was added and the solution was brought to 37°; then 0.1 ml of enzyme (0.01 mg) was added and maintained at this temperature for 3 min (incubation for 1 min gave maximum inhibition). To the latter solution 4.0 ml of cold buffer were added, and 0.1 ml of the resulting solution was mixed with 2.0 ml of a reaction mixture containing 0.33 M sodium pyruvate, 0.067 mM NADH, and 0.027 M phosphate buffer at pH 7.4. Rate = change in absorbance per minute/6.22 × milligrams of enzyme per milliliter of reaction mixture. Controls representing 100% activity were made by substituting buffer or 0.15 mM CPZ for irradiated CPZ solutions.

Time solution held	Inhibition	
	2°	40°
min	%	%
0	69	62
8	66	57
30	58	57
1200	62	33
2880	66	32

TABLE 4

Changes in inhibitory activity toward lactate dehydrogenase of irradiated CPZ solutions maintained at pH 4 at 2° and 40°

The irradiated solution was adjusted to pH 4 with 1.0 N HCl and maintained at 2° or 40° for the indicated times. At the end of each interval, 1 volume of the irradiated solution was mixed with 0.9 volume of 0.15 M phosphate buffer at pH 7.4. This was brought to 37°; 0.1 ml of enzyme was added, the solution was held at 37° for 3 min and diluted, and the enzyme activity was tested as described in Table 3.

Time solution held	Inhibition	
	2°	40°
min	%	%
0	89	87
55	84	88
225	86	92
1620	86	80
2880	84	75
4320	88	98
5760	89	87

had not diminished significantly after 4 days at 40°.

The blue-green and pink residues from 100 ml of 0.15 M irradiated CPZ were each dissolved in 2.0 ml of 0.08 M phosphate buffer, pH 7.4, diluted, and maintained in the dark for varying periods of time at 2° or 40°. At the end of these periods 0.2-ml aliquots of the solutions were diluted with 1.7-ml quantities of buffer and brought to 37°. A 0.1-ml portion of the enzyme solution was added to each portion of the inhibitor solution, the enzyme-inhibitor solution was incubated for 3 min at 37°, and 4 ml of cold buffer were added. One-tenth-milliliter portions of these solutions were used to initiate assays of lactate dehydrogenase activity. As shown in Tables 5 and 6, the inhibitory effects of the solutions of the blue-green and pink residues were less affected by the higher temperature than was the inhibition by irradiated CPZ. The solution of the blue-green residue had changed to a yellow color when it was held at 40° for 60 min, and the solution of the pink residue had become yellow after 18 hr at this temperature. EPR signal strength in the solutions of the residues changed during incubation, but the inhibitory activity of these solutions remained unchanged (see Tables 5 and 6).

TABLE 5

Changes in EPR signal strength and inhibitory activity toward lactate dehydrogenase of solutions of blue-green residue at pH 7.4

The blue-green residue from 100 ml of 0.15 mM irradiated CPZ was dissolved in 2.0 ml of 0.08 M phosphate buffer at pH 7.4; portions of these solutions were maintained in darkness at the temperatures indicated. At an appropriate time, 50 μl of the solutions at 40° were tested for EPR signal strength. For the test of inhibitory activity, 0.2 ml was taken from solutions at 2° and 40° and mixed with 1.7 ml of 0.15 M phosphate buffer at pH 7.4, and the solution was brought to 37° for 3 min; 4.0 ml of cold buffer were added, and 0.1 ml of this solution was added to the test solution described in Table 3. EPR signal strength is expressed as a percentage of signal strength at zero time, immediately after dissolution of the residue.

Time solution held	Inhibition		Relative strength of EPR signal
	2°	40°	
min	%	%	%
0	58	67	100
60	60	64	200
180	61	65	30
300	58	67	26
1140	64	70	15

TABLE 6

Changes in EPR signal strength and inhibitory activity toward lactate dehydrogenase of solutions of pink residue at pH 7.4

Measurements of EPR signal strength and inhibitory activity of solutions of the pink residue were accomplished by the methods described in the legend to Table 5.

Time solution	Inhibition		Relative strength of EPR signal
	2°	40°	
min	%	%	%
0	70	57	100
150	71	58	72
960	69	59	70
1440	62	59	64

The effect of irradiated CPZ on the activity of UDP-glucose dehydrogenase (uridine diphosphate glucose:NAD^+ oxidoreductase, EC 1.1.1.22) (Sigma) was measured by incubating 0.5 ml of a 5 mg/ml solution of the enzyme with 1.5 ml of the irradiated CPZ solution at 36° for 3 min. Equal volumes of buffer and irradiated CPZ solution served as controls. After this preliminary incubation, 1 ml of a solution of 1.8 mM NAD^+ and 2.7 mM UDP-glucose in 0.15 M phosphate buffer, pH 7.4, was added to initiate the reaction. The rate of reduction of NAD^+ was observed in terms of the increase in absorbance at 340 nm during the first 3 min of the reaction. Incubation with 0.075 mM irradiated CPZ produced 79% inhibition; incubation with CPZ under the same conditions resulted in no inhibition of UDP-glucose dehydrogenase activity.

A similar study was carried out using crystalline rabbit muscle glyceraldehyde 3-phosphate dehydrogenase [D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12] (Sigma).

To 0.1 ml of a solution of CPZ, irradiated CPZ, or buffer, 0.02 ml of a 0.9 mg/ml solution of glyceraldehyde 3-phosphate dehydrogenase was added. After incubation at 36° for 3 min, 2 ml of a solution of 0.24 mM DL-glyceraldehyde 3-phosphate, 0.102 mM NAD^+, 0.026 M sodium pyrophosphate, and 0.014 M disodium arsenate were added, and the rate of reduction of NAD^+ at 36° was followed in terms of the change in absorbance at 340 nm. Incubation with 0.015 mM irradiated CPZ produced 97% inhibition, whereas incubation with the same concentration of CPZ had no effect on the activity of glyceraldehyde 3-phosphate dehydrogenase.

DISCUSSION

Irradiation of dilute, neutral solutions of CPZ by ultraviolet light at 253.7 nm resulted in a decrease of the CPZ absorption band at 255 nm and the formation of a new band at 248 nm, indicating the transformation of CPZ to other products. EPR examination showed the presence of free radicals in the irradiated solutions. Extraction of the irradiated solutions with heptane yielded a blue-green residue from the heptane fraction and a pink residue from the aqueous phase after evaporation. Heptane extraction of solutions irradiated at pH 4 did not yield the blue-green product; however, evaporation of the aqueous phase again gave a pink residue. The blue-green and pink residues were found to contain different free radicals when dissolved in aqueous solutions at pH 7.4.

The action of inhibitors in dilute irradiated solutions of CPZ was studied with yeast alcohol dehydrogenase, an enzyme that contains about 40 reactive sulfhydryl groups per molecule. The inactivation of the enzyme was strongly dependent on temperature and on the concentration and duration of exposure to the inhibitor. Thus exposure to 0.015 mM irradiated CPZ at 36° for 1 min did not result in inhibition, whereas exposure under identical conditions for 1.5 and 2 min resulted in a marked diminution of alcohol dehydrogenase activity. Exposure of the enzyme to 10-fold higher concentrations of irradiated CPZ for 1 min at 36° abolished activity completely, whereas exposure for 10 min at 2° produced only 72% inhibition.

The stability of compounds that inhibit enzyme activity was studied with lactate dehydrogenase. The activity of irradiated solutions of CPZ at pH 7.4 was unchanged after storage for 2 days at 2°, but decreased by 50% after storage for 2 days at 40°. At pH 4 the activity of the solutions was essentially unchanged after storage for 4 days at 40°. During the maintenance of solutions of the blue-green and the pink residues at 2° or 40° for extended periods, their inhibitory properties changed little. Thus, contrary to the observation of Wollemann (9, 18), stable inhibitors were present both in unfractionated dilute irradiated solutions of CPZ and in the fractions prepared from them.

The absorption and EPR spectra of the solutions of the residues and of the irradiated CPZ solutions were different from the spectra of the CPZ semiquinone free radical. Also, the semiquinone radical is unstable at neutral pH, in contrast to the free radicals from the irradiated solutions. Thus, under the conditions employed in this study, it appears improbable that the semiquinone free radical contributed to the inhibition observed. Certainly, if such free radicals were formed by irradiation, they would be expected to act as enzyme inhibitors. Lagercranz (19) and others studied the EPR spectra of solutions of CPZ irradiated at near neutrality and detected relatively stable paramagentic species, but these did not show EPR spectra characteristic of the chemically produced free radical of CPZ.

To interpret previous studies, one must consider the contribution of relatively stable photolytic products to the mechanism of enzyme inhibition by irradiated CPZ solutions at pH 7.4. Although some evidence for stable free radicals derived from CPZ solutions irradiated at this pH has been reported briefly by Piette (20), Van Woert (21), Lagercranz (19), and Blois (22), the activity of these free radicals as enzyme inhibitors appears not to have been studied. Several reactions of CPZ initiated by irradiation at near neutrality are known to occur, producing exceedingly

short-lived reactive species that do not include the semiquinone free radical. When a protein is present in the CPZ solution during irradiation, these reactions may produce a modification of protein structure, and could conceivably inactivate enzymes. In one such reaction, photoexcited CPZ was shown to form covalent bonds with serum albumin. This reaction appears to take place through photonucleophilic aromatic substitution of the chloride ion (23, 24). Another mechanism by which the irradiation of CPZ could inactivate enzymes is the oxidation of sulfhydryl groups by an oxygen-dependent photodynamic action. Yet this reaction occurs also only during the period of irradiation (25).

We have demonstrated that CPZ can be transformed into relatively stable free radicals, and that irradiated solutions of CPZ can directly inactivate enzymes even after these solutions have been stored in the dark for long periods of time. Solutions of the residues from the aqueous and heptane fractions also contained products that persisted for several days and that inhibited the activity of sulfhydryl-dependent enzymes, although the free radical signals decayed during this time. Neither signal strength, character of the EPR spectra, nor color of solutions of the residues could be related in a simple manner to the inhibitory properties of these solutions. Thus, although relatively stable free radicals distinct from the chemically produced semiquinone free radical can be demonstrated in solutions of the fractions prepared from dilute irradiated CPZ solutions, these stable free radicals, which may or may not possess a semiquinone structure, do not appear to have been responsible for the enzyme inhibition observed.

It appears likely that the inhibition of enzyme activity reported by Akera and Brody (26) and by Levy and Burbridge (12) resulted from the action of long-lived irradiation products as well as short-lived photoexcited species of CPZ other than the semiquinone free radical. The evidence that relatively long-lived, biochemically active compounds are formed by ultraviolet irradiation of CPZ solutions at physiological pH may be of importance in understanding the occurrence of oculocutaneous pigmentation during CPZ treatment, and in explaining the mechanisms of the psychopharmacological effects of CPZ.

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