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INHIBITION OF URIDINE DIPHOSPHATE GLUCOSE DEHYDROGENASE* BY A FREE RADICAL FORMED FROM CHLORPROMAZINE

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Abstract—That inhibition *in vitro* of uridine diphosphate glucose dehydrogenase by chlorpromazine (CPZ) might depend upon oxidation of CPZ to a free radical was suggested when preincubation of the enzyme with CPZ in daylight produced inhibition of the enzyme coincident with oxidation of the CPZ. Chlorpromazine-5-oxide, the oxidation product, did not inhibit the enzyme, nor did CPZ without preincubation. The inhibitory species seems, therefore, to be the CPZ free radical, an intermediate in the oxidation of CPZ to its oxide.

Generation of the free radical has been accomplished by oxidation of CPZ with Ce^{4+}, and by solution of CPZ or its oxide in strongly acidic solutions. The mechanism of free radical generation has been studied, and a stable CPZ free radical preparation which may be conveniently employed as an inhibitor of enzymes *in vitro* has been developed.

Preformed CPZ free radical is a potent inhibitor of uridine diphosphate glucose dehydrogenase. Inhibition is noncompetitive with respect to both the substrate and the coenzyme. Cysteine protects the enzyme from inhibition, while neither the substrate nor the coenzyme affords protection.

MANY reports[1–47] dealing with the inhibition *in vitro* of enzyme systems by chlorpromazine (CPZ) and other phenothiazines contain indirect evidence that some transformation of CPZ is required for inhibitory activity. The time occupied by preincubation, temperature equilibration, and the assay of enzyme activity itself afforded an opportunity for transformation of the compound by the enzyme preparation. In several reports, either inhibition was progressive[9, 18, 24, 29]—that is, the degree of inhibition increased during the course of the assay, or inhibition was produced or increased by deliberate preincubation of the compound with the enzyme preparation.[4, 11, 29, 40, 42, 44, 45]

Wollemann and her co-workers[48, 49] reported that CPZ completely inhibited the activity of a crystalline phosphoglyceraldehyde dehydrogenase preparation when the drug was preincubated with the enzyme in daylight; the drug did not produce inhibition unless it was preincubated with the enzyme preparation; and preincubation in the dark or in the light in the presence of cysteine did not result in inhibition. These authors suggested that a free radical formed from CPZ by photo-oxidation in the presence of the enzyme was the actual inhibitory species.

* Uridine diphosphate glucose: NAD^+ oxidoreductase, EC 1.1.1.22.

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This present study was launched when preliminary experiments demonstrated the failure of CPZ to inhibit another NAD^+-linked oxidoreductase—uridine diphosphate glucose (UDPG) dehydrogenase—and suggested the possibility that a one-electron oxidation of CPZ to its free radical was required for inhibition of this enzyme.

Many workers[50–70] have prepared the CPZ free radical and have studied its chemistry. It has been necessary to study further the preparation of the CPZ free radical and some of its properties in order to prepare the free radical in a form convenient for the study of the inhibition of an enzyme preparation *in vitro*. The preformed CPZ free radical has been demonstrated to be a potent inhibitor of UDPG dehydrogenase.

METHODS AND RESULTS

Generation of the CPZ free radical

Absorption spectra and difference spectra were recorded by means of a Beckman DK 2A ratio-recording spectrophotometer. The concentrations of CPZ and of CPZ-5-oxide in the same solution were estimated graphically from the absorption spectrum of each solution.[71] Since the proportionality between the ESR signal and the absorbance at 525 mμ has been demonstrated,[61, 63–65] measurements of absorbance sufficed to measure concentrations of the CPZ free radical.

Initially, the free radical prepared according to the method described by Wollemann and Keleti[49] yielded an absorption spectrum (Fig. 1) suggesting a mixture of

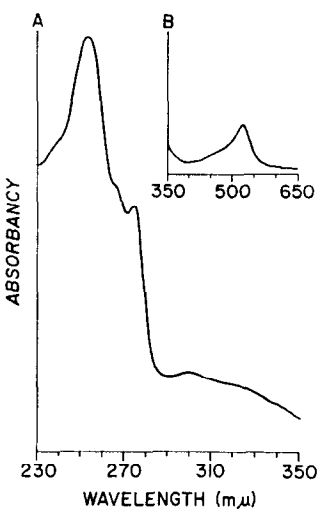

FIG. 1. Ce^{4+} oxidation of CPZ in dilute acid. The absorption spectrum is that of a 1:10,000 dilution of the reaction mixture in 1 N H_2SO_4.

CPZ and the oxide in the proportion of 3:1, along with the CPZ free radical in a yield of about 7·5 per cent.

Following the suggestion of Tuck,* we attempted production of the free radical in various concentrations of HCl. No free radical resulted when equimolar amounts of

* Personal communication.

CPZ and the oxide as their hydrochloride salts were dissolved in distilled water or in 2 N HCl. The yield of free radical was 20 per cent in 4 N HCl and approximately 60 per cent for concentrations of HCl equal to or greater than 6 N. The rate of free radical generation, indicated by the rate of increase of absorbance at 525 mμ, increased with increasing [H^+]. Solution of CPZ and the oxide in 8 N HCl resulted almost instantaneously in the appearance of a substance absorbing maximally at 525 mμ, and giving the absorption spectrum for the CPZ free radical described by Borg and Cotzias.[60] The rate of increase of the absorbance at 525 mμ seemed greatest when equimolar amounts of CPZ and the oxide were used, and was appreciably slower when the reaction mixture contained a large excess of one component or the other. The eventual yield of the free radical varied only with the [H^+] but not with the proportion of CPZ. Important differences were noted among various batches of HCl in the course of these experiments. Free radical production from CPZ occurred at a much slower rate when once-distilled and de-oxygenated HCl was employed as a solvent.

Because of instability of the CPZ free radical in HCl, its production in H_2SO_4 was studied. As in HCl, the rate of free radical formation increased with increasing [H^+]. And the rate was greater for free radical production from an equimolar mixture of CPZ and the oxide than from either substance alone.

Because Borg and Cotzias[61] and Tozer[69] had demonstrated that Ce^{4+} was capable of the complete oxidation of CPZ, this oxidant was chosen for the production of the CPZ free radical. Portions of a stock ceric ammonium sulfate [$NH_4Ce(SO_4)_4 \cdot 2H_2O$] solution in 19 N H_2SO_4 were added to a $1{\cdot}5 \times 10^{-5}$ M CPZ solution in the same solvent. There was absorption at 525 mμ before the addition of oxidant. The absorbance at 525 mμ was maximal after the addition of 1·5 equivalents of Ce^{4+} and could still be detected after the addition of 6 equivalents of the oxidant. Addition of Ce^{4+} beyond the 2-equivalent point resulted in spectral evidence of a new compound (Fig. 2). This spectrum was observed in a variety of circumstances: (1) when the

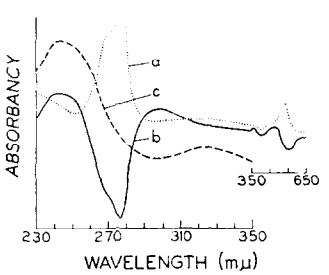

FIG. 2. Ce^{4+} oxidation of CPZ in 19 N H_2SO_4. The spectra are as follows: a, difference spectrum: CPZ + 1 equivalent of Ce^{4+} minus CPZ in acid alone; b, difference spectrum: CPZ + 4 equivalents of Ce^{4+} minus CPZ + 1 equivalent of Ce^{4+}; c, absorption spectrum: CPZ + 8 equivalents of Ce^{4+}.

spectrum resulting from the oxidation of CPZ with 2 equivalents of Ce^{4+} was subtracted from that resulting from the oxidation of CPZ with 4 equivalents of Ce^{4+} in 19 N H_2SO_4; (2) when 8 equivalents of oxidant were added to CPZ in 19 N H_2SO_4; and (3) when 6 equivalents of oxidant were added to CPZ-5-oxide dissolved in 19 N H_2SO_4. This oxidation product, apparently possessing eight fewer electrons than

CPZ, gave a faint yellow color. Spectra similar to these are given in the report of Felmeister and Discher[68] for the product of the irradiation of CPZ with light at 253·5 mμ.

In 12 N H_2SO_4, free radical production was maximal when 1·5 equivalents of Ce^{4+} had been added. Further additions of the oxidant resulted in the appearance of a spectrum similar to that resulting from the solution of the oxide in 12 N H_2SO_4. In 6 N H_2SO_4, maximal absorbancy at 525 mμ was never achieved. The spectrum most characteristic of the free radical was noted after the addition of 2·5 equivalents of the oxidant. Further additions of Ce^{4+} resulted in the spectrum of the oxide.

Determination of the molar absorbance of the free radical. In order to measure the molar absorbance of the CPZ free radical at 525 mμ, the diperchlorate hemihydrate salt of the free radical was prepared by a modification of the procedure of Merkle and his co-workers.[67] Approximately 1·4 m-mole of CPZ hydrochloride were dissolved in 10 ml of 70% $HClO_4$, after which 0·08 ml of 30% H_2O_2 was added. The resulting intensely colored solution was diluted with 10 ml acetone, followed by 20 ml ether. After the solution had been permitted to stand in the freezer for a few hours, the resulting purple-black crystals were filtered, washed with ether, and dried. A small amount of the crystalline material was dissolved in 50% H_2SO_4, and the absorbance at 525 mμ of the resulting solution was determined to be $1{\cdot}04 \times 10^4$ l/mole × cm.

Preparation of the CPZ free radical stock solution. A stock free radical solution was prepared by the solution of 48 mg of CPZ hydrochloride and 50·1 mg of CPZ-5-oxide hydrochloride ($1{\cdot}35 \times 10^{-4}$ mole of each) in 1 ml of 16 N H_2SO_4. A more dilute stock solution was prepared by dilution of a portion of this stock solution with an equal volume of 16 N H_2SO_4. The absorbance of these solutions at 525 mμ became maximal after standing for 24 hr and remained unchanged thereafter for months at room temperature. That conversion of CPZ and the oxide to the free radical was complete was demonstrated by comparison of the measured absorbance at 525 mμ with the molar absorbance for the CPZ free radical.

Enzyme studies

Lots of UDPG dehydrogenase† possessing a sp. act. of 160,000–200,000 units/g‡ were employed.

Enzyme assay. The assay of enzyme activity was adapted from that of Strominger *et al.*[72, 73] Approximately 50 mg of the lyophilized enzyme preparation containing 0·31 mg protein/mg (according to the method of Waddell[74]) was dissolved in 10 ml of ice water. A 0·5-ml aliquot of this stock enzyme solution was added to 1 ml phosphate buffer (I = 0·15, pH 7·4) in a quartz cell with a 1-cm light path; 0·15–1·0 ml of a solution of NAD^+ (Sigma) ($1–2 \times 10^{-3}$ M) was added, followed by 1 ml or less of a solution of the inhibitor. The volume was then adjusted to 3 ml by the addition of water. The quartz cell was allowed to equilibrate with a temperature of 30 ± 0·5° for 5 min in the cell compartment of the recording spectrophotometer equipped with a time-drive attachment and a thermostatically regulated cell holder. The assay was

† Purchased from the Sigma Chemical Co., St. Louis, Mo.

‡ A unit of enzyme activity is that quantity of enzyme producing a change of absorbance of 0·001/min at 340 mμ.

then initiated by the addition of 2–50 μl of a solution of UDPG (Sigma) ($7{\cdot}5$–30×10^{-3} M). This was accompanied by vigorous stirring after which the absorbance at 340 mμ was recorded as a function of time. The initial reaction velocity was measured from the record of the change of absorbance.

All assays of enzyme activity were performed in duplicate and in random order.

Enzyme kinetics. Preliminary studies of the reaction kinetics of this enzyme preparation yielded an apparent K_m for UDPG of $1{\cdot}1 \times 10^{-5}$ M ($[NAD^+] = 6 \times 10^{-4}$ M), and for NAD^+ of $1{\cdot}4 \times 10^{-4}$ M ($[UDPG] = 10^{-4}$ M). Maxwell and coworkers reported corresponding values of 2×10^{-5} M and $1{\cdot}1 \times 10^{-4}$ M.[72]

Effect of CPZ. CPZ produced no inhibition of enzyme activity at a final concentration of 2×10^{-5} M, whereas 9 per cent inhibition was produced by a final concentration of 10^{-4} M. CPZ-5-oxide in a final concentration of 10^{-4} M produced no inhibition whatever.

Preincubation of the enzyme with CPZ required modification of the assay: $1{\cdot}5$ ml of the stock enzyme solution was diluted with 3 ml of the phosphate buffer; $1{\cdot}5$ ml of the CPZ solution (or water, for control assays) was then added, and the buffered enzyme solutions were permitted to stand in daylight at room temperature for approximately 30 min. Then 2-ml portions of these solutions were placed in quartz cells to which 1-ml portions of an NAD^+ solution (10^{-3} M) had been added. After temperature equilibration, the reaction was initiated by the addition of UDPG in the usual fashion. Preincubation of the buffered enzyme solution in the absence of substrate with 10^{-4} M CPZ in daylight produced 31 per cent inhibition. Analysis of the difference spectrum between a 10-fold dilution of the assay solution and a similar

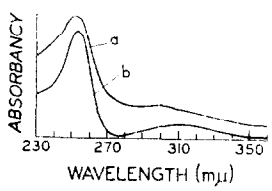

FIG. 3. Difference spectra of CPZ-containing assay solutions with and without preincubation in the light. Spectrum a is the difference between the 1:10 dilution of the assay solution preincubated with CPZ in daylight and a similar dilution of the control assay solution to which no CPZ had been added. Spectrum b is the difference between the dilution of the assay solution to which CPZ had been added immediately prior to assay and the diluted control assay solution.

dilution of the control assay solution (containing no CPZ) revealed that the preincubated solution contained approximately equimolar concentrations of CPZ and the oxide (Fig. 3). A similar analysis revealed that no oxide was present in the assay solution to which CPZ had been added without preincubation. Thus, in the solution to which CPZ was added without preincubation and in which only negligible inhibition occurred, the CPZ was not transformed, whereas the CPZ was oxidized and inhibition produced in the solution preincubated with CPZ.

Enzyme inhibition by the CPZ free radical. The study of the action of the free radical formed from CPZ on the activity of the enzyme also required modification of the

assay. In a preliminary study, the addition of 1 μl 16 N H_2SO_4 to 3 ml of assay solution reduced the pH of the solution from 7·4 to 7·0, at the same time reducing enzyme activity significantly. Thus, because the stock free radical solution could be maintained only at this very high [H^+], it was possible to add only 1 μl of this solution to each final 3-ml volume of assay solution in order not to exceed the capacity of the buffer.

In a preliminary experiment, the free radical in a final concentration of $4{\cdot}5 \times 10^{-5}$ M produced 36 per cent inhibition when compared to two control assays. In the first control assay, 16 N H_2SO_4 was substituted for the stock solution of the free radical. In the second, the stock free radical solution was added to the buffer and

TABLE 1. INHIBITION OF UDPG DEHYDROGENASE BY CPZ FREE RADICAL

Enzyme solution	v*
1	0·020
2	0·028
3	0·033

Enzyme preparation (53·8 mg) was dissolved in 13 ml water, yielding a protein concentration of 1·33 mg/ml.[71] Enzyme solutions were prepared as follows:
(1) 1·5 ml stock enzyme solution was diluted with 3 ml phosphate buffer (pH 7·4); 3 μl free radical solution was then added;
(2) 1·5 ml stock enzyme solution was diluted with 3 ml buffer; 3 μl 16 N H_2SO_4 was then added;
(3) 3μl free radical solution was placed in flask; 3 ml buffer was then added and the flask swirled until the color had disappeared; 1·5 ml enzyme solution was then added;
1·5 ml of each enzyme solution was transferred to quartz cells, to which were then added 1 ml NAD^+ (2×10^{-3} M) and 0·5 ml water, and the reactions were then initated by the addition to each cell with mixing of 20 μl UDPG ($1{\cdot}5 \times 10^{-2}$ M).
* v = initial reaction velocity, measured as the change in absorbance at 340 mμ/min.

permitted to decay *before* the enzyme solution was added. The data from this experiment are presented in Table 1.

In a further experiment, inhibition was studied as the concentration of free radical was varied from 1·5 to 9×10^{-5} M. The results of this experiment, plotted in Fig. 4, suggest that lower concentrations of the free radical are not inhibitory, whereas somewhat higher concentrations are markedly inhibitory.

The inhibition produced by the free radical in a final concentration of 6×10^{-5} M was next studied as the concentrations of the substrate (UDPG) and coenzyme (NAD^+) were varied. Lineweaver–Burk plots[75] prepared from these experiments are presented in Figs. 5 and 6. The free radical may be seen to inhibit the enzyme noncompetitively with respect to each substrate, with an apparent K_i of $3{\cdot}7 \times 10^{-5}$ M.

For the study of the protection afforded the enzyme by the addition of various substances to the buffered enzyme solution prior to the addition of the free radical solution, a similar assay was employed. Portions (1·5 ml) of the solutions to be tested for their ability to protect the enzyme from inhibition were added to 1·5-ml portions of the stock enzyme solution diluted with 3 ml of the phosphate buffer.

Two μl of a 0·27 M solution of the free radical in 16 N H_2SO_4 were then added to the buffered enzyme solutions. After these solutions had become colorless, 2-ml portions were transferred to quartz cells. After the addition to each cell of 1 ml of water (or of NAD^+, in the study of protection by cysteine), temperature equilibration was

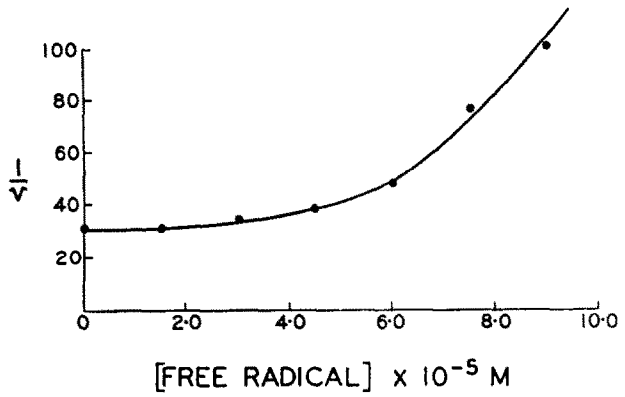

FIG. 4. Inhibition of UDPG dehydrogenase by CPZ free radical: initial reaction velocity vs. [free radical]. Fifty-three mg of the enzyme preparation was dissolved in 11 ml water, yielding a protein concentration of 1·6 mg/ml. Working enzyme solutions were prepared by the addition to 1·5 ml portions of this stock enzyme solution of 3 ml of phosphate buffer and a total of 3 μl of one or more of the following solutions: 16 N H_2SO_4, free radical (0·135 M), free radical (0·27 M); 1·5 ml NAD^+ ($1·3 \times 10^{-3}$ M) was added to 1·5-ml portions of the working enzyme solutions, and the reactions were initiated by the addition with stirring of 10 μl UDPG (2×10^{-2} M).

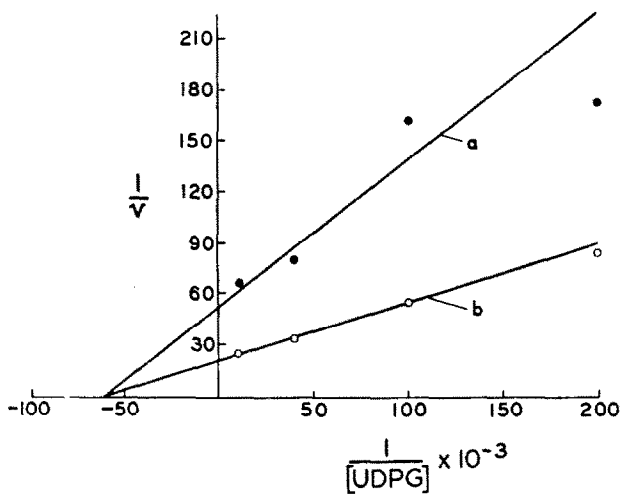

FIG. 5. Inhibition of UDPG dehydrogenase by CPZ free radical: initial reaction velocity vs. [UDPG]. [NAD^+] 6×10^{-4} M; a, [Free radical] 6×10^{-5} M; b, no inhibitor.

permitted to occur, and the reactions were initiated by the rapid addition, with mixing, of 20-μl portions of solutions of the missing component—NAD^+ in the study of protection by UDPG, and UDPG in the study of protection by both NAD^+ and cysteine.

The experiments summarized in Table 2 considered the protection of the enzyme from inhibition by the CPZ free radical. UDPG, NAD^+, and cysteine were tested for their ability to prevent or minimize inhibition when these substances were added to the buffered enzyme solution prior to addition of the free radical. Neither substrate nor coenzyme protected the enzyme from inhibition by the CPZ free radical, whereas

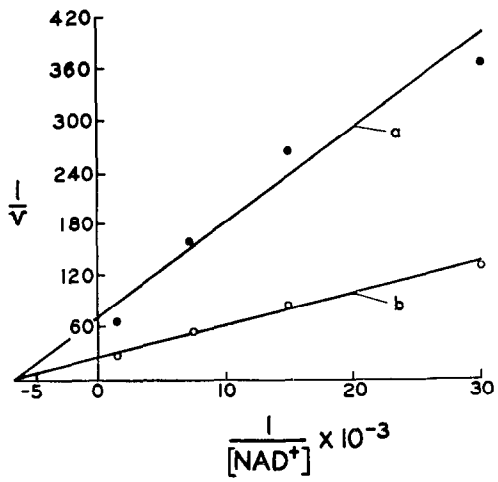

FIG. 6. Inhibition of UDPG dehydrogenase by CPZ free radical: initial reaction velocity vs. $[NAD^+]$. [UDPG] 10^{-4} M; a, [free radical] 6×10^{-5} M; b, no inhibitor.

TABLE 2. INHIBITION OF UDPG DEHYDROGENASE BY CPZ FREE RADICAL: PROTECTION OF THE ENZYME

Substances added*	% Inhibition
Enzyme + free radical (6×10^{-5} M)	47
Enzyme + cysteine ($2{\cdot}5 \times 10^{-4}$ M) + free radical	16
Enzyme + free radical + cysteine	38
Enzyme + NAD^+ (6×10^{-4} M) + free radical	47
Enzyme + UDPG (10^{-4} M) + free radical	47

* Substances are listed in the order of their addition.

cysteine provided considerable protection. Cysteine also produced partial restoration of enzyme activity when it was added after the free radical had been permitted to react with the buffered enzyme.

DISCUSSION

When the UDPG dehydrogenase preparation employed in these studies was found not to be directly inhibited by CPZ, preincubation of the enzyme with CPZ was undertaken. It was then possible to demonstrate that oxidation of CPZ to the oxide occurred during preincubation of CPZ with the enzyme in daylight coincident with inhibition of the enzyme. CPZ-5-oxide, in a concentration twice that produced during preincubation, was shown not to be inhibitory in a separate experiment. It appeared likely, therefore, that the CPZ free radical, intermediate in the oxidation of CPZ to the oxide, was the actual inhibitory species.

In an attempt to confirm this hypothesis, preparation of the free radical was undertaken. The low yield which resulted from the attempt to prepare the CPZ free radical by the method of Wollemann and Keleti[49] suggests that the CPZ initially present in the reaction mixture was indeed oxidized to the free radical, but that the free radical then underwent decay to CPZ and the oxide.

The mechanism of free radical generation from CPZ in acid alone remains speculative. It seems likely that the oxidation of CPZ to the free radical was carried out by contaminants present in the acid, particularly dissolved O_2 and Cl_2 in the case of free radical generation from HCl. Evidence for this may be found in the decreased rate of free radical formation in redistilled and deaerated HCl. Free radical formation in acid alone from the oxide or from mixtures of CPZ and the oxide is more readily explained. In a sufficiently acid medium, the CPZ phenazothionium ion, formed from the oxide,[62] is capable of yielding the free radical and another product at a level of oxidation higher than that of the phenazothionium ion.

At least three equations must be written to describe free radical formation in acid:

$$\text{CPZ-5-oxide} + H^+ \rightleftharpoons \text{phenazothionium}^+ + H_2O \quad (1)$$

$$\text{phenazothionium}^+ + \text{CPZ} \rightleftharpoons 2 \text{ free radical} \quad (2)$$

$$2 \text{ phenazothionium}^+ \rightleftharpoons \text{free radical} + \text{oxidized product} \quad (3)$$

At a sufficiently high $[H^+]$, reaction (1) occurs very rapidly. When the free radical is generated from the oxide, or from a mixture of CPZ and the oxide, reactions (2) and (3) would seem to be rate-limiting. The overall reaction might be expected to proceed very slowly when the free radical is generated from CPZ alone. From these considerations, one might expect the formation of the free radical in acid alone to be most rapid from an equimolar mixture of CPZ and the oxide, and to be slowest from CPZ alone. This was indeed the case for free radical generation in HCl. In H_2SO_4, on the other hand, free radical generation was slowest from the oxide alone.

Generation of the free radical by Ce^{4+} oxidation in strong acid was resorted to in an effort to solve some of the stoichiometric problems encountered when free radical generation was carried out in acid alone. It was quickly apparent that the equilibria described by equations (1), (2), and (3) resulted from preparation of the free radical by Ce^{4+} oxidation of CPZ. The problem was further complicated by the appearance of a new spectrum observed when the oxidation was carried out at a high $[H^+]$. These studies suggest that, because of disproportionation of the free radical and of the phenazothionium ion, these compounds in solution are always present in an equilibrium mixture, the composition of which varies with the $[H^+]$ and with the quantity of oxidant added.

In order to measure the molar absorbance of the free radical so that the kinetic measurements could be plotted and the rate constants calculated, it was necessary to prepare a crystalline salt of the free radical which could be separated from the other components of the equilibrium mixture. Because the free radical disproportionates when the salt is dissolved in strong acid, resulting in some equilibrium mixture, the measured molar absorbance may well represent an underestimation of the true molar absorbance. The measured value, on the other hand, may represent the best estimate, since in separating the salt it was possible to purify the free radical of those components of the equilibrium mixture that might oxidize or reduce the free radical.

As in Wollemann's experiments, the free radical solution was found to inhibit directly, without need for preincubation. That the inhibition resulted from an action of the free radical on the enzyme protein rather than on some other component of the assay system seems clear. The free radical was usually added to a solution containing only buffer and enzyme, and had completely decayed or reacted with the enzyme protein before any of the other components of the assay system was added. Furthermore, inhibition of enzyme activity by the free radical was of the same degree, whether or not either substrate or coenzyme was present at the time of addition of the free radical to the buffered enzyme solution.

A study of the inhibition produced by the free radical prepared from CPZ revealed the inhibition to possess the following characteristics: (1) inhibition was noncompetitive with respect to both substrate and coenzyme; (2) neither substrate nor coenzyme protected the enzyme from inhibition; (3) cysteine protected the enzyme preparation from inhibition when it was added prior to the addition of the free radical and produced partial restoration of activity when it was introduced after addition of the free radical to the buffered enzyme solution; (4) the degree of inhibition was not proportional to the concentration of the free radical, but was minimal in the presence of concentrations of the free radical smaller than 6×10^{-5} M, and became marked in concentrations of the free radical greater than this.

These observations suggest that when the preformed free radical is added to the enzyme, inhibition results from an attack on the enzyme molecule by the free radical. It is tempting to speculate that the free radical exerts its inhibitory effects by means of a titration of important functional groups, as, for example, sulfhydryl groups.

There is insufficient evidence from which to conclude that the inhibition resulting from preincubation with CPZ was accomplished by a mechanism similar to that by which the preformed free radical produced inhibition. Preformed free radical added to a solution of the enzyme may be free to attack the enzyme molecule at points not available to the free radical generated by photo-oxidation. The free radical is exceedingly reactive and does not exist for very long at a neutral pH. It seems unlikely, therefore, that the free radical generated by photo-oxidation in the enzyme solution buffered to pH 7·4 would survive long enough to diffuse through the solution to attack the enzyme at sites distant from its site of generation.

In apparent contrast to the studies reported here is the work of Khouw *et al.*,[76] who found CPZ to be strongly inhibitory to both horse-liver and rabbit-liver alcohol dehydrogenase. Preincubation of the enzyme with CPZ performed in the dark at 4° had no effect on the inhibition, which was instantaneous in character. Khouw,[77] by means of polarized fluorescence measurements, observed that CPZ complexed with the protein-bound NAD^+ (or NADH) in the course of its inhibition of alcohol dehydrogenase. The possibility remains, however, that the CPZ free radical may be inhibitory at concentrations even smaller than those at which unchanged CPZ was found to produce inhibition. Yagi and his co-workers,[78] working with what may be an analogous system, found that CPZ formed a complex with the flavin coenzyme of D-amino acid oxidase, and that CPZ inhibited the enzyme competitively with respect to the coenzyme. These authors concluded, however, that this was not the mechanism by which CPZ inhibited the enzyme,[18] since inhibition (which appears to be progressive) could be demonstrated at a CPZ concentration one-fiftieth that of the concentration required for complex formation. Since the assay employed by Khouw *et al.*

required measurement of the reaction velocity only during the first 30 sec of the reaction, there was not the opportunity to observe whether progressive inhibition occurred.

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