guishable from the synthetic hydroxyl compound by dextran adsorption chromatography (Fig. 1) and by migration in Solvents D–F (Table I). Compound V could not be detected as a metabolite.

Another newly identified metabolite is unconjugated IV. It is assumed tentatively that the second ring hydroxyl group is in the same position as in III and that the diol metabolite may be formed from this intermediate and from II.

One cannot be certain about the oxidation of II or IV *in vivo*. Nevertheless, these conversions are postulated in Scheme III because II was oxidized readily *in vitro* by human and rat erythrocytes (7). In view of the β-adrenoceptor blocking activity of both I and II (6), it seems reasonable to speculate that III and IV are active metabolites and, therefore, to consider that I is not only an active compound but a prodrug which is converted to three active metabolites.

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Effects of Adenine Nucleotides on Oxidation of Phenothiazine Tranquilizers

IRA BLEI

Abstract □ The effects of adenosine diphosphate and triphosphate on the periodic acid oxidation of the phenothiazine tranquilizing drugs were studied. The principal effect was a marked reduction in the rate of formation and decay of the drug free radical. The oxidation rates of the nucleotide free drugs seemed to be most strongly influenced by the inductive effects of substituents at the 2-position of the phenothiazine nucleus. However, the oxidation rates of the drugs in the presence of nucleotide were most strongly influenced by the substituents at the 10-position. Variations of the structure of substituents at the 10-position have only a modest effect on the electronic state of the phenothiazine nucleus. Therefore, the marked effect of structural variation in the 10-substituents in the presence of nucleotide on the periodate oxidation rate most likely is an expression of steric effects related to an interaction between drug and nucleotide.

Keyphrases □ Adenosine diphosphate and triphosphate—effect on periodic acid oxidation of various phenothiazines □ Phenothiazines, various—periodic acid oxidation, effect of adenosine diphosphate and triphosphate □ Oxidation—various phenothiazines by periodic acid, effect of adenosine diphosphate and triphosphate □ Nucleotides—adenosine diphosphate and triphosphate, effect on periodic acid oxidation of various phenothiazines □ Tranquilizers—various phenothiazines, periodic acid oxidation, effect of adenosine diphosphate and triphosphate

This study was based on an effect observed during a fluorescence quenching study of chlorpromazine (I)–adenosine triphosphate (II) complex formation, previously studied by surface chemical methods (1) and later verified by spectroscopic techniques (2). In the presence of nucleotides, the phenothiazine drug oxidation rates caused by UV irradiation were markedly reduced compared with nucleotide free drug systems. It was felt that a study of this effect might reveal useful details regarding the nucleotide–drug interaction. The general objective of this work was not to obtain absolute rate constants or other kinetic parameters but rather to obtain relative reaction rates from oxidation systems identical with respect to all variables except for structural variations of a group of phenothiazine tranquilizing drugs.

EXPERIMENTAL

Qualitative and quantitative analyses for phenothiazine free radicals were performed principally by spectrophotometry. The identity of the free radical was verified by parallel electron spin resonance measurements (3). The products of oxidation were characterized by TLC, spot tests (4), and UV–visible spectrophotometry (5).

To use spectrophotometry for quantitative analysis, the initial drug concentration had to be about 1×10^{-3} *M*. The system then had to be poised with respect to both nucleotide and oxidant concentrations. The nucleotide concentration was made high enough to assure maximal free radical formation. The periodic acid concentration was not too low so that too small a fraction of drug would be converted to semiquinone nor too high so that the reaction would proceed too rapidly for adequate quantitation. A typical final reaction mixture was: drug, 1×10^{-3} *M*; II, 1×10^{-2} *M*, tromethamine (III)–hydrochloride buffer, pH 7.0, 2×10^{-2} *M*; and periodic acid, 1×10^{-2} *M*.

A typical experiment was performed as follows. Drug, nucleotide, and buffer were added to a volumetric flask and diluted to a precalibrated mark with distilled water. An aliquot of periodic acid stock solution was then added to begin the reaction, followed by final adjustment of volume. Under these conditions, the final pH ranged from 3.0 to 4.0.

Molar absorptivities were determined by periodic acid oxidation of

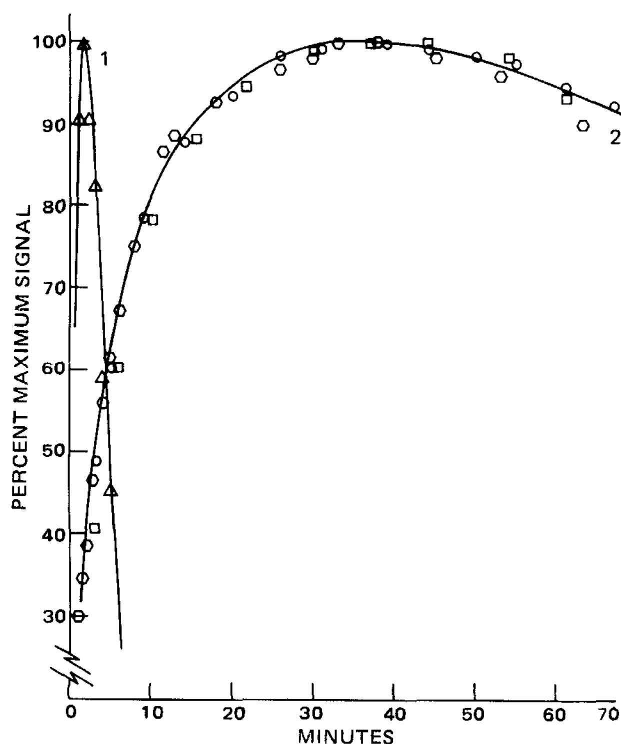

Figure 1—*Time course of periodic acid oxidation of chlorpromazine with and without adenosine triphosphate. The reaction mixture contained: drug, 1 × 10^{-3} M; periodic acid, 1 × 10^{-2} M; pH 7.0 buffer, 2 × 10^{-2} M; and II, 1 × 10^{-2} M. Key:* △, *absorbance at 525 nm, drug alone;* ○, *absorbance at 525 nm, drug plus II; and* ○, □, *electron spin resonance signals, drug plus II.*

the drugs in 2 *M* H_2SO_4 (3). The log of absorbance varied linearly with time. Furthermore, different concentrations of periodic acid resulted in lines with correspondingly different slopes, all converging on the same absorbance value when extrapolated back to zero time. The zero-time absorbance values were used to compute the molar absorptivities. The value of the I free radical obtained in this way compares well with previously published data (6). The absolute values of absorptivities for the drug free radicals were not critical for comparison of initial reaction rates. This comparison required only that the values were correct relative to each other, and the approach described was sufficient to that end.

The drugs in this study were chlorpromazine[1], promazine[1], trifluoperazine[1], fluphenazine[2], triflupromazine[2], and thioridazine[3]. Nucleotides II and adenosine diphosphate (IV)[4] were used without purification. All other materials were reagent grade. Spectrophotometric measurements[5] and electron spin resonance spectra[6] were taken at room temperature.

RESULTS AND DISCUSSION

Phenothiazine oxidation is considered to proceed in acid media through two consecutive steps (7):

$$H^+ + R = S^+ + e \qquad \text{(Eq. 1)}$$

$$S^+ = T^+ + e \qquad \text{(Eq. 2)}$$

where R represents the totally reduced form of the drug, S^+ is the free radical or semiquinone, and T^+ is the totally oxidized phenazathionium cation. Depending upon pH and concentration, T^+ can react with water to form either sulfoxide or aromatic hydroxylated derivatives.

Over a wide range of experimental conditions and types of drugs and nucleotides, the initial stage of the oxidation process resulted only in the formation of semiquinone. The second stage of oxidation, semiquinone

[1] Smith Kline and French.
[2] E. R. Squibb.
[3] Sandoz.
[4] Sigma Chemical Co.
[5] Hitachi-Coleman model 124 double-beam spectrophotometer.
[6] Varian E3 ESR spectrometer.

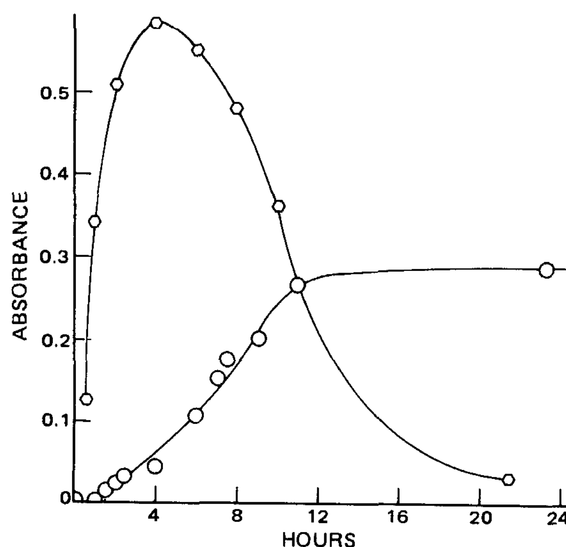

Figure 2—*Time course of promazine oxidation by periodic acid in the presence of II. The reaction mixture contained: promazine, 1 × 10^{-3} M; periodic acid, 5 × 10^{-3} M; II, 1 × 10^{-2} M; and pH 7.0 buffer, 2 × 10^{-2} M. Key:* ○, *absorbance of the drug free radical at 510 nm; and* ○, *twice the absorbance of iodine at 515 nm in carbon tetrachloride. Iodine was extracted from reaction mixtures by shaking an aliquot of reaction mixture with an equal volume of carbon tetrachloride.*

decay in the presence of nucleotide, resulted in complex mixtures of iodine and dehalogenated, demethylated, and variably hydroxylated phenothiazine derivatives. The work therefore focused on the initial reaction of the oxidation process—*viz.*, semiquinone formation, for two reasons. First, that approach minimized analytical and kinetic complexities. Second, since the effects of nucleotide on both free radical formation and decay probably reflected the same underlying molecular mechanism, little information would be lost by not studying the decay process.

The likelihood of complexation was presumed to be maximized in the pH range from 2.0 to 7.0, where the drug was cationic and the nucleotide was anionic. It would have been most desirable to poise the system as close to pH 7.0 as possible. However, at about that pH, several complicating phenomena occur: formation of neutral (insoluble) drug base, formation of drug micelles dependent upon salt type and concentration, and formation of insoluble periodic acid salts. By employing the described conditions, the system was optimized with respect to the electrostatic state of the drug and nucleotide and solution homogeneity.

The reaction of periodate with the phenothiazines is illustrated by two types of experiments in Fig. 1. Curve 1 represents the periodic acid oxidation of I measured spectrophotometrically; curve 2 represents the same system, with II added, measured both spectrophotometrically and by electron spin resonance spectroscopy. In all cases, the data are presented as percent of maximum signal of the free radical and absorbance:

$$\%\text{ maximum signal} = \left(\frac{\text{observed signal}}{\text{maximum signal}}\right) 100 \qquad \text{(Eq. 3)}$$

The assumption was that both the absorbance and intensity of the electron spin resonance signal were linearly related to free radical concentrations at the drug concentration used. The periodic acid oxidation of drug in nucleotide-free systems, as illustrated by curve 1 of Fig. 1, was typically biphasic, with the rapid appearance of the free radical followed by its equally rapid decay. Under these conditions, the oxidation appeared to be complete within about 10 min. The presence of II dramatically altered the rates of formation and decay of the free radical, as illustrated by curve 2 of Fig. 1. The absorbance and electron spin resonance data fell on the same curve, satisfying the assumptions concerning the relationship of the free radical concentration to both absorbance and the electron spin resonance signal and also permitting the legitimate use of absorbance as a measure of the free radical concentration in these systems. The addition of II had a marked stabilizing effect on the I free radical. Similar effects were observed when IV replaced II.

As the reaction proceeded, other striking differences were observed between the pure drug and nucleotide-containing systems (Fig. 2). The free radical-stabilizing effects of II were best illustrated by the oxidizing system employing promazine, since this drug was the least stable phe-

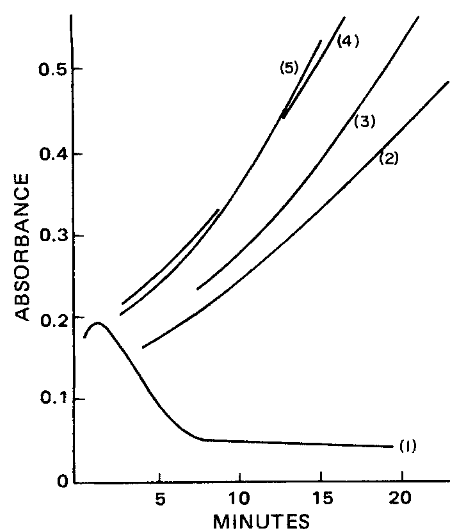

Figure 3—*Time course of oxidation of chlorpromazine in the presence of varying quantities of II. The reaction mixture contained: drug, 1 × 10^{-3}* M; *periodic acid, 5 × 10^{-3}* M; *pH 7.0 buffer, 2 × 10^{-2}* M; *and no II (curve 1), 5 × 10^{-5}* M *II (curve 2), 1 × 10^{-4}* M *II (curve 3), 2 × 10^{-4}* M *II (curve 4), or 3 × 10^{-4}* M *II (curve 5).*

nothiazine. The upper biphasic curve represents the appearance and decay of the promazine free radical in the presence of II. The periodate concentration was half that of the system illustrated in Fig. 1. The maximum concentration of the free radical occurred after ~4 hr. Under the same conditions without II, the maximum free radical concentration was reached in about 5 min and totally decayed in about 20 min.

The lower curve traces the appearance of iodine. For ease of visualization, the iodine concentrations are presented as double their actual values. Only in the drug–nucleotide systems is iodine a product of the drug–periodate reaction. In nucleotide-free, drug–periodate systems, the oxidant is reduced to iodate and no iodine is formed. The time course of iodine generation is typical for that of the final product of a two-step consecutive reaction system, here probably two consecutive oxidation steps. Furthermore, although detailed analyses of the drug oxidation products were not undertaken, spot tests and TLC indicated the presence of demethylated, dehalogenated, and aromatic hydroxylated products in the systems containing nucleotides. However, only the sulfoxide was produced in the nucleotide-free systems.

Control experiments showed that the nucleotide had no effect on the

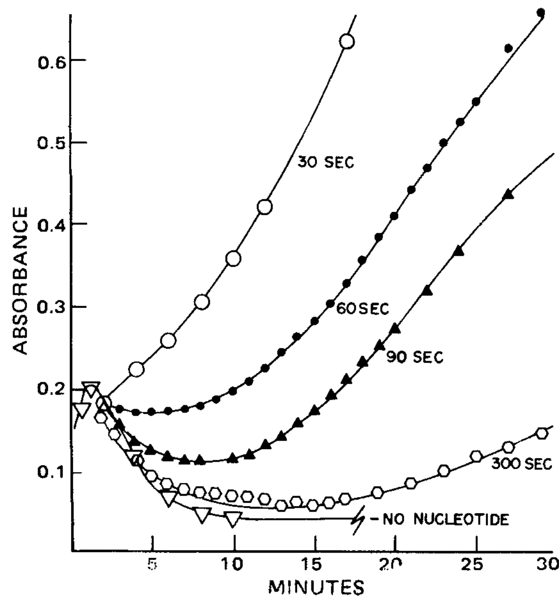

Figure 4—*Time course of oxidation of 1 × 10^{-3}* M *promazine by 1 × 10^{-2}* M *periodic acid in 2 × 10^{-2}* M *pH 7.0 buffer. Curves are labeled to represent elapsed times of reaction prior to addition of II sufficient to bring the solutions to 1 × 10^{-2}* M *with respect to nucleotide.*

Table I—Initial Rates of Drug Free Radical Formation *versus* Drug Potency [a]

Drug	Rate of Formation × 5 ± 0.5 × 10^7, moles/liter/min: With IV	Without IV	E_m ± 0.1 E_m, liters/mole cm	Average Antipsychotic Dosage (9), mg
Promazine	875	7000	1 × 10^4 (510 nm)	750
Chlorpromazine	250	1750	1 × 10^4 (525 nm)	550
Thioridazine	65	4850	1.5 × 10^4 (625 nm)	300
Triflupromazine	40	—	5 × 10^3 (500 nm)	200
Trifluoperazine	17	630	9.5 × 10^3 (500 nm)	9
Fluphenazine	6	1320	5 × 10^3 (510 nm)	6

[a] A typical reaction mixture contained: drug, 1 × 10^{-3} *M*; adenosine diphosphate (IV), 1 × 10^{-2} *M*; III-hydrochloride pH 7.0 buffer, 2 × 10^{-2} *M*; and periodic acid, 1 × 10^{-2} *M*.

periodic acid oxidation of sodium metabisulfite, sodium sulfite, aniline, and *o*-phenylenediamine. Furthermore, exposure of II to 0.1 *M* periodic acid for 24 hr had no effect on the nucleotide's ability to stabilize the drug free radical. The effects of II concentration on the initial rate of periodate oxidation of chlorpromazine are illustrated in Fig. 3. Curve 1 represents a nucleotide-free system in which free radical rapidly forms and decays. Curves 2–5 represent similar systems with increasing II concentrations.

These differences in oxidation rates and the nature of products between systems with and without nucleotide are best explained as consequences of nucleotide–drug complex formation. In an earlier study, intercalation or complexation also seemed to provide the basis for the ability of deoxyribonucleic acid to stabilize the I free radical (8). This finding posed the next question: if a complex was formed, was the complex oxidized directly or did the free drug arise from complex dissociation.

This question was probed by the following experiments (Fig. 4). Compound II was added as dry powder to buffered promazine–periodic acid systems at 30, 60, 90, and 300 sec after the oxidation was begun. For reference purposes, the lowest curve is a system without nucleotide; the free radical concentration rose to a maximum in about 1 min and then began to decay. From the data in Fig. 4, it is clear that the sooner nucleotide is added to the drug–oxidant system, the greater is the extent of stable free radical formation. An alternative view is that the extent of stable free radical formation and/or accumulation depends upon the concentration of unoxidized drug. The first oxidation step in the drug–nucleotide systems therefore appears to be subsequent to a process requiring both the presence of nucleotide and unoxidized drug—*viz.*, complex formation.

It seemed desirable to attempt to correlate the effects of nucleotide on drug oxidation with some pertinent biological data. The specific approach was to obtain initial oxidation rates of drug–nucleotide systems under identical experimental conditions, varying only the nature of the drug. The goal was to determine whether or not a reasonable relationship between these oxidation rates and the "clinical effectiveness," *i.e.*, human drug dosage for a specific behavioral end-point, could be established. Although the specific values for drug dosage (9) may be controversial, the relative values of dose *versus* drug type seem well established. Therefore, it was considered satisfactory to use these values to explore the relationship between the oxidation rate of the drug–nucleotide complex and drug structure. Accordingly, the results of experiments designed to obtain these data along with experimental conditions are presented in Table I and Fig. 5. Reference to Fig. 5 indicates an apparent correlation of initial oxidation rates of drug–nucleotide systems with clinical dosages. There appears to be no correlation of initial oxidation rates of nucleotide-free drug systems with drug dosages (Table I).

The differences in oxidation rates among the different drug–nucleotide systems may be viewed as reflecting differences in the degree of steric hindrance to oxidation as a result of complex formation. Figure 6 illustrates the manner in which nucleotide and drug might interact to form a complex.

In Fig. 6A, space-filling models of II and chlorpromazine are poised in a relationship allowing an approach favoring complexation. In Fig. 6B, the approach is complete and shows a close complementary fit. There appears to be ample opportunity for salt formation between phosphate groups of II and the quaternized terminal nitrogen of the drug's pendant 3-(*N*,*N*-dimethylamino)propyl group. As a result of this apposition, the hydrophobic moieties approach in such a way as virtually to "bury" the

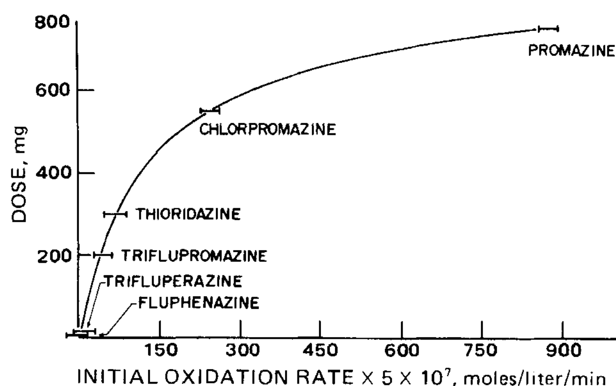

Figure 5—*Initial rates of phenothiazine drug free radical formation in the presence of IV as a function of drug potency. The rates were arbitrarily scaled for convenient comparison of values. Experimental details may be found in Table I.*

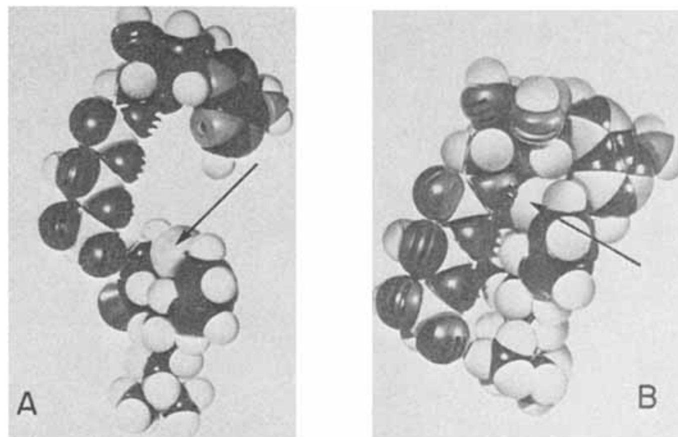

Figure 6—*Photographs of space-filling models. Key: A, II and chlorpromazine as they might approach in solution; and B, view of a possible model of the resulting complex. The arrows point to the ring sulfur atoms for reference.*

sulfide sulfur within the domain of the complex (see arrows) and to hinder severely the approach of an oxidant, particularly one as bulky as periodate. Complexation should also reduce the electrostatic attraction between the drug cation and the oxidant anion.

Reference to Fig. 6 shows that the plane of the phenothiazine nucleus is normal to the plane formed by the purine and ribose–phosphate moieties of the nucleotide. The substituent at the 2-position is removed from the area of contact between the two molecules. Therefore, it should have only an electronic inductive effect on the oxidation rate rather than a steric effect.

Promazine and chlorpromazine differ in structure only at the 2-position. Reference to Table I indicates a significant effect on the rates of drug oxidation by the nucleotide. However, the ratios of promazine oxidation rate to that of chlorpromazine in the presence and absence of nucleotide are the same to within ±7%. This result indicates that the inductive effects on the oxidation rate caused by substituents at the 2-position continue to operate, apparently unmodified by complexation, along with the superposition of steric effects caused by substituents at the 10-position.

Substituents at the 10-position are intimately involved in the proposed complex, and differences there should be reflected in the extent to which oxidation rates are modified. Fluphenazine, trifluoperazine, and triflupromazine have identical substituents at the 2-position but differ in structure at the 10-position. They demonstrated significant differences in drug oxidation rates in the presence of nucleotide, not related simply to their oxidation rates in nucleotide-free systems (Table I). However, the extent of oxidation rate reduction for these three drugs in the presence of nucleotide did seem to be related to the dimensions of the substituents at the 10-position; the larger the dimensions, the greater was the reduction in the oxidation rate. These facts are consistent with the proposal that drugs and nucleotide interact to form a sterically hindered reductant.

The experimental systems described required drug concentrations much higher than would ever be encountered in an *in vivo* situation. Even so, the general relationships demonstrated with this model system would not be significantly altered by decreasing drug concentrations to those comparable to the *in vivo* situation where nucleotide concentrations may be orders of magnitude greater than those of the drugs.

The relationship described in Fig. 5 is consistent with the view that, within a family of similarly acting drugs, differences in effectiveness are related to differences in absorption rates, distributions, and metabolic fates. In this particular case, the data indicate that the differences may be principally related to metabolic fate: oxidation. Furthermore, the clinical or apparent effectiveness of the phenothiazine tranquilizers did not correlate with the oxidation rate of free drug but rather of a drug–nucleotide complex. Implicit in this finding is the possibility that the drugs may exist within the cell to a significant extent in the form of drug–nucleotide complexes. The data presented here support the probability of the *in vitro* existence of such complexes.

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