

# 6 | Oxidative susceptibility testing

Paul Harmon and Giovanni Boccardi

## INTRODUCTION

Water and molecular oxygen (dioxygen) are the two ubiquitous molecules that most frequently affect the stability of a drug substance. Though acids and bases are the main catalysts that control the hydrolytic behavior of organic compounds, they are not the principal factors in oxidations. In many cases, oxidation involving dioxygen is hard to understand and may also seem difficult to reproduce. This difficulty is compounded by consideration of dioxygen as a potential reactant with organic substrates. In the orbital diagram of molecular oxygen, the highest occupied molecular orbitals are two degenerate  $\pi^*$  orbitals in which there must be two electrons (Fig. 1). The ground state, according to the Hund rule, is the state in which these orbitals are occupied by one electron and the spins are parallel: this is the triplet ground state ( $^3\Sigma_g^-$ ) of atmospheric molecular oxygen (Fig. 1). However, the vast majority of organic molecules are in the singlet state, and the reaction:



is spin-forbidden. For this reason, a large number of organic molecules, in spite of the large negative value of the Gibbs free energy of oxidation, are kinetically inert toward dioxygen.

How then does oxidation of drug molecules in formulated solid dosage forms proceed? A significant clue is the common experience of many pharmaceutical scientists, in which a pure drug substance is quite stable toward oxidation, but in the formulated drug product the drug substance oxidatively degrades. One significant contributor to this effect is the role that excipient impurities often play as “initiators” of oxidation (1–4). As will be described below, these impurities can give rise to peroxy and alkoxy radicals  $\text{R}_{\text{imp}}\text{OO}\bullet$  and  $\text{R}_{\text{imp}}\text{O}\bullet$  which are actually the species that initially react with drug molecule carbon–hydrogen (D–H) bonds. These reactions produce drug molecule radicals ( $\text{D}\bullet$ ) which by virtue of their unpaired electron are in a triplet state, and thus are very reactive with dioxygen. This simple concept explains how the spin forbidden reaction (1) is readily obviated and accounts for variable formulation and batch-dependent oxidation rates often encountered by pharmaceutical scientists.

The aim of this chapter then is to provide the reader with the necessary theoretical framework to understand most oxidations one is likely to encounter and to review the current methodologies, tests, and accepted practices to predict to what extent drug molecules will be oxidatively sensitive. The first edition of *Pharmaceutical Stress Testing* (5) did not consider several more recently described methodologies aimed at examining oxidative liability of drug molecules. These include methodologies based on DSC (6), cyclic voltammetry (7,8) and a novel type of peroxy radical-based stressing system (9). While the latter two of these will be discussed in some detail, an additional focus of this chapter will be to incorporate more recent work on practical experimental aspects such as oxygenation requirements, solvent choice and pH effects for the two most common oxidative susceptibility tests—azonitrile initiation and the hydrogen peroxide test. The goal of these methodology optimizations is to ensure that drug substances are exposed to the appropriate oxidants during the stress test.

In section “Mechanistic Background for the Most Common Oxidation Routes,” we will review the mechanistic background for three oxidation routes. The first is autoxidation, by far the most common oxidative route, which is “initiated” as described above and involves a radical chain process. The second route is oxidation of drug molecules by organic hydroperoxides and/or hydrogen peroxide in a nonradical reaction, in which all electrons are paired. Finally, oxidation mediated by single electron transfer (SET) to dioxygen will be considered. In section “Practical Tests and Considerations for Oxidative Susceptibility Testing,” we will review practical experimental aspects of solution phase oxidative susceptibility testing. Finally, in section “Summary and General Strategy of Oxidative Susceptibility Testing,” we will provide a brief overall strategy of oxidative susceptibility testing.



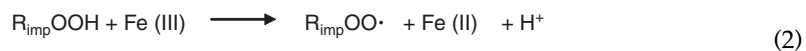
**Figure 1** Electronic configurations of molecular oxygen: triplet ground state and the singlet first excited state. The figure shows the highest occupied molecular orbitals.

## MECHANISTIC BACKGROUND FOR THE MOST COMMON OXIDATION ROUTES

### Autoxidation

#### Autoxidation Mechanism

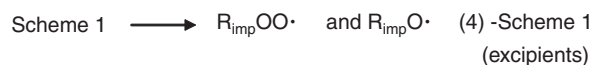
The mechanism of autoxidation has been studied extensively in solution and reviewed (1,2,10). While some minor differences between solution and solid state (oral dosage form) autoxidation might be expected, the solution-based understanding of autoxidation provides the basis for describing autoxidation in the solid state. A brief overview follows. Autoxidation is described in three stages: initiation, propagation, and termination. In the current context, we chose to specifically highlight the role that excipient impurities can play in the initiation stage of autoxidation in solid dosage forms. By far, the most common excipient impurities which are capable of initiating oxidation are organic hydroperoxides ( $R_{\text{imp}}\text{OOH}$ ). Organic hydroperoxides are found at trace levels (hundreds to thousands of nanomoles/g excipient) in numerous excipients such as Tween 80, PEG 400, HPMC, PVP, and others, and have been well studied (11–13). Hydroperoxides can be catalytically degraded by trace levels of transition metal ions (in particular Fe(III), which is ubiquitous) to form equimolar amounts of peroxy and alkoxy radicals as shown in Scheme 1:



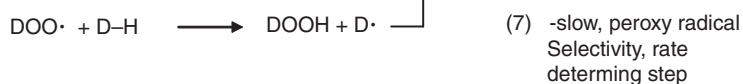
**Scheme 1** Catalytic cycle for Fe(III)/(II) and an excipient-related hydroperoxide  $R_{\text{imp}}\text{OOH}$ .

Given this initiation step, the subsequent propagation and termination steps for autoxidation of hydrogen bearing  $\text{sp}^3$  carbon atoms of a drug molecule (D–H) is shown in Scheme 2:

#### Initiation



#### Propagation



#### Termination



**Scheme 2** Autoxidation mediated by drug-based peroxy radical ( $\text{DOO}\cdot$ ).

The initiation of oxidation of the drug molecule is shown in Eqs. (5a) and (5b) as abstraction of drug H-atoms from D–H bonds by the impurity-derived radicals. Both Eqs. (5a) and (5b) generate D• radicals. Given the known large rate constant for oxygen reaction with carbon radicals, which is near  $10^9 \text{ M}^{-1}\text{S}^{-1}$  or higher at 300 K (14) and the general availability of dissolved oxygen in solution or oxygen gas in the case of a solid dosage form, Eq. (6) shows that the primary reaction expected is formation of drug-derived peroxy radicals DOO•. Equation (7) is the key reaction in autoxidation, the reaction of the drug-derived peroxy radical with the drug itself. Peroxy radical reactions are relatively slow [for example, the cumene rate constant  $k_p$  is  $0.18 \text{ M}^{-1}\text{s}^{-1}$  at 303 K (10)] and are thus typically the rate-limiting step in Scheme 2. Both products in Eq. (7) are important. The first, DOOH, is the drug hydroperoxide and represents the first stable or metastable oxidation product one might identify either in a solution or an oral dosage form. The second product in Eq. (7) is another drug radical D•, which again will oxygenate (upward arrow in Scheme 2) to give another drug peroxy radical as shown in Eq. (6). This repeating process [Eq. (6) to Eq. (7) to Eq. (6), etc.] is referred to as peroxy radical chain propagation or just propagation. For “oxidatively sensitive” drugs, the amount of drug oxidized in Eqs. (6) and (7) can be expected to be significantly larger than the drug oxidized in the initiation steps Eqs. (5a) and (5b). Finally, the termination reaction in Eq. (8) occurs when the D–H concentration begins to diminish enough that drug peroxy radicals encounter each other and react to give non-radical products [mechanism is detailed below in Eq. (9)]. In an oral dosage form, the D–H concentration decrease could be viewed as a local phenomenon such as degradation in a defect zone or in an amorphous region of an otherwise crystalline lattice.

Some further discussion on the selectivity of the key peroxy radical reaction in Eq. (7) is warranted. Peroxy radical C–H bond reaction rates are related to the substrate’s C–H bond dissociation energy in comparison to that of the peroxy radical ROO–H bond energy, which is about 89 kcal/mole (1,2,10). Thus substrate C–H bonds with bond energies less than 89 kcal/mole may react relatively rapidly with peroxy radicals while higher bond energy C–H bonds will react much slower. Compilations of C–H bond energies and reaction rates with peroxy radicals are available (15) and can be useful in understanding potential C–H bond reactive sites. Most C–H bond energies are significantly larger than 89 kcal/mole which accounts for the selectivity of peroxy radical reactions. In addition, peroxy radical may also undergo addition reactions with olefin bonds (not shown in Scheme 2) which will have all the same mechanistic outcomes as shown in Scheme 2. Many drug molecules will have only very slow (if any) reaction with peroxy radicals, while others may have significantly faster reaction rates. This difference, in the context of Scheme 2, is what makes one drug substance “oxidatively stable” while another is “oxidatively sensitive” given similar initiation rates in Eqs. (5a) and (5b).

We will not go in depth into the subject of antioxidants used in pharmaceutical formulations (for reviews, see Refs. 1–3,16). It is worthwhile however to note from Scheme 2 why the autoxidation mechanism can often be effectively inhibited by phenolic antioxidants such as butylated hydroxyl anisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate. These compounds have two unique properties: (i) very low energy O–H bonds which can rapidly donate hydrogen atoms to the propagating peroxy radicals in Eqs. (6), (7), and (ii) the resulting antioxidant radicals are not reactive with molecular oxygen themselves, being stabilized through delocalization and steric hindrance (16). These “chain breaking antioxidants” work particularly well if the effective peroxy radical chain propagation length is long since “quenching” of one peroxy radical in this way dramatically reduces the amount of drug subsequently oxidized. The fact that BHA and BHT are commonly and successfully used to stop oxidation in oral dosage forms suggests that even in the solid state, propagation chain lengths are significantly greater than one.

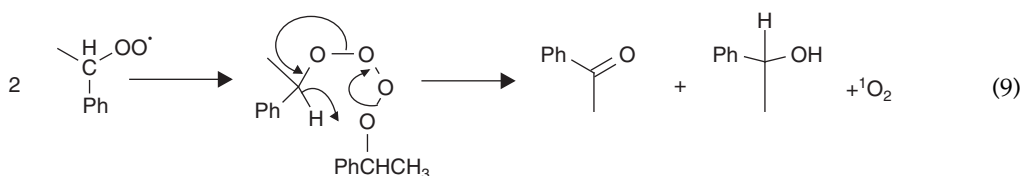
This chapter does not deal with the kinetics of oxidative degradation, treated elsewhere in detail (17), because the authors believe degradation kinetics is a more advanced task in drug development than the oxidative susceptibility stress test. One aspect of the kinetic treatment of Scheme 2 should be highlighted, however. Expressions for the rate of autoxidation in solution are proportional to the product of the substrate concentration and the rate constant of the

peroxy radical hydrogen atom abstraction [Eq. (7), Scheme 2]. Typically, there is no observed rate dependence on dissolved oxygen concentrations for solutions in equilibrium with ambient oxygen (0.21 atmospheres) given that the rate constant for Eq. (6) is much larger than the rate constants for Eq. (7) in Scheme 2 (18,19). Oxygenation will be briefly revisited in section "The Fate of the Unstable Peroxy Species: The Origin of the Stable Degradation Impurities."

*The Fate of the Unstable Peroxy Species: The Origin of the Stable Degradation Impurities*

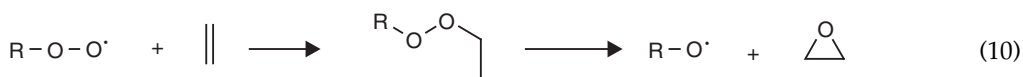
The degradation profile from Scheme 2 will reflect the peroxy radical chemistry in Eqs. (6)–(8). Hydroperoxides are the primary degradation products of autoxidation and can be found as degradation impurities, but the most stable products develop in side reactions involving hydroperoxides and peroxy radicals. Some of these processes surrounding the formation and decomposition of hydroperoxides will be summarized here.

- *Termination reactions.* A very common termination reaction, known as the Russell mechanism from its discoverer, is the recombination of two peroxy radicals to form an unstable tetroxide that decomposes through a concerted mechanism to yield an alcohol moiety and a carbonyl (20):



The oxygen molecule produced by this mechanism is in the singlet state. Equation (9) is shown for a secondary benzylic peroxy radical, but is general for primary and secondary peroxy radicals.

- *Epoxide formation.* Peroxy radicals can react with carbon–carbon double bonds to produce epoxides (21):

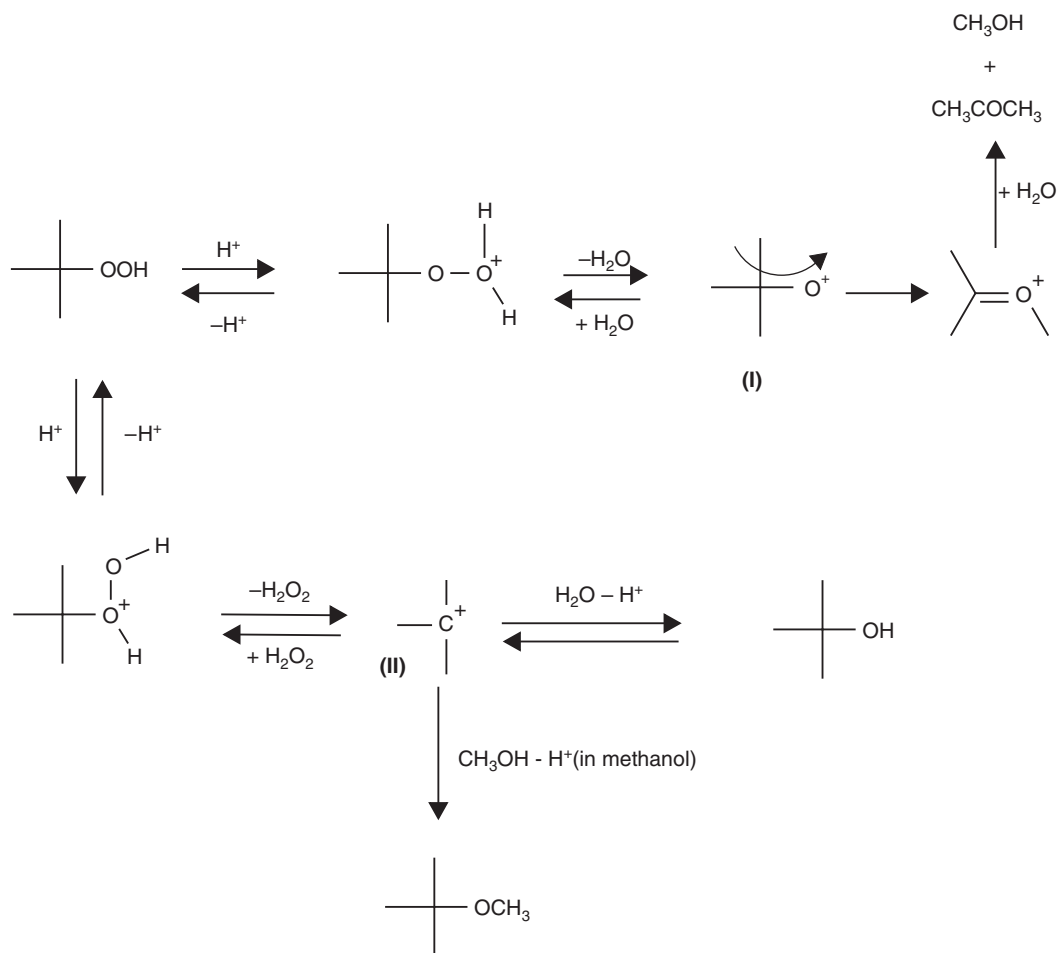


This reaction introduces the concept of co-oxidation in that the alkoxy radical  $\text{RO}\cdot$  produced in Eq. (10) may also oxidize substrates in addition to peroxy radicals.

- *Acid decomposition.* Hydroperoxides are decomposed by acids (Fig. 2). The first step is protonation, and two paths are possible for the protonated hydroperoxide. The first is elimination of a water molecule, giving the oxonium ion(I) that can rearrange, yielding an alcohol and a ketone. This path is favored by substituents on the carbon atoms that can migrate. A well-known example is cumene oxidation, which is the basis of the industrial phenol-acetone process. The second path is the elimination of hydrogen peroxide to yield the carbocation(II) in Figure 2. The carbocation can add a nucleophile such as water or an alcohol, or other carbocation scavengers. Carbocations can also rearrange causing ring expansion, or elimination of a proton to give a carbon–carbon double bond (22).

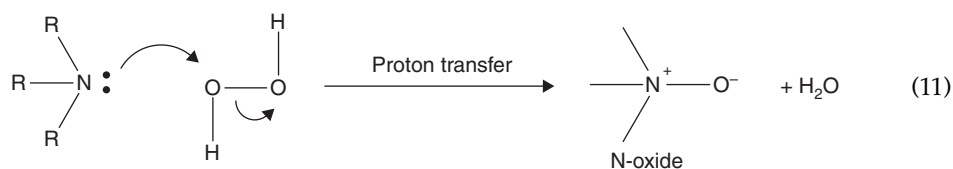
**Oxidation by Organic Hydroperoxides and Hydroperoxide**

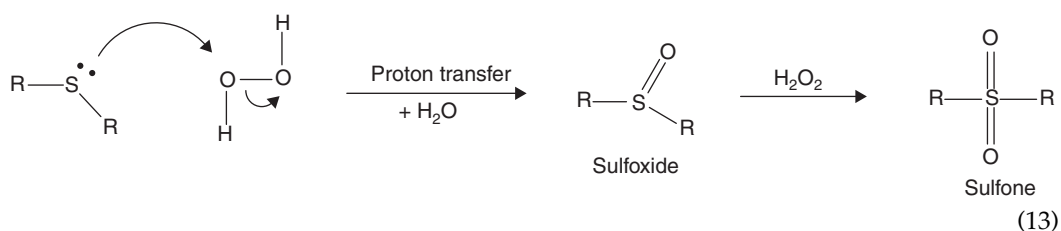
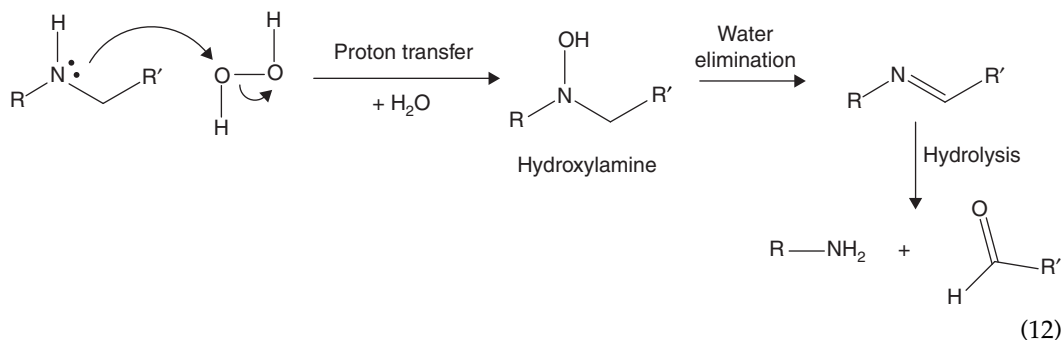
Hydroperoxides ROOH are the first metastable product formed in autoxidation as shown in Scheme 2. Hydrogen peroxide can be produced by elimination during acid decomposition of hydroperoxides as shown in Figure 2. Thus, reaction of drug molecule functional groups with hydroperoxides needs to be explicitly considered and this represents a distinct oxidation



**Figure 2** Acid decomposition of hydroperoxides (22): main pathways. Dehydration and rearrangement (*upper*), loss of  $\text{H}_2\text{O}_2$ , and nucleophilic attack of the solvent (*bottom*).

pathway from autoxidation. In this context, we are interested in reactions which may occur under long-term storage conditions; that is, at a maximum of  $30^\circ\text{C}$  or  $40^\circ\text{C}$ . Equations (11)–(14) show the most common reactive groups of drug molecules. Considering the intact hydroperoxide as the oxidizing reagent, Eqs. (11) and (12) show electrophilic attack on tertiary and secondary amines to form the *N*-oxide and hydroxylamine, respectively. The tertiary amine reaction is generally more favorable. Equation (12) shows further decomposition of the hydroxylamine for completeness. Note that the amine reactions will have a marked pH dependence in Eqs. (11) and (12); the reactions being much slower in the protonated state. Equation (13) shows electrophilic attack on a thioether to give a sulfoxide and then further a sulfone:



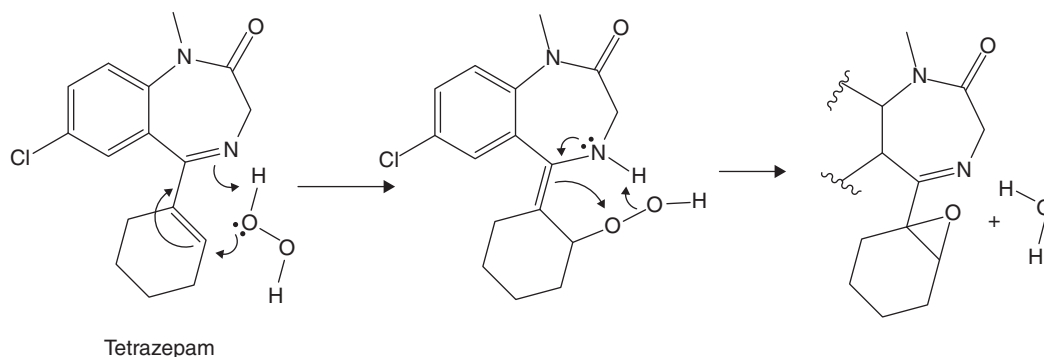


Equation (14) shows the epoxidation of a carbon–carbon double bond. This reaction is typically much slower than Eqs. (11)–(13) and is more appropriately viewed as a nucleophilic attack of the hydroperoxide on the olefin bond. In Eqs. (11)–(14), all the reactions are “ionic” in that all electrons are paired, radicals are not involved:



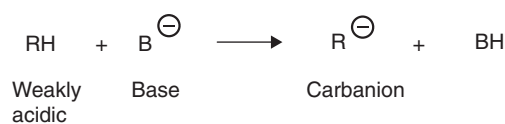
The epoxidation reaction is worthy of further discussion. Under the mild conditions described here and discussed further below in section “Practical Tests and Considerations for Oxidative Susceptibility Testing,” reaction (14) proceeds at a reasonable rate only if there is some sort of stabilizing/supporting structural effects associated with the R and R' groups which facilitate the attack of the relatively weak nucleophile (hydrogen peroxide). Tetrazepam offers a likely case example of this type of effect, where the tetrazepam epoxide is obtained in fairly high yield from dilute hydrogen peroxide solution in methanol or acetonitrile at 40°C (23). Figure 3 shows how tetrazepam likely facilitates the nucleophilic attack and epoxide formation.

While more reactive reagents such as peracetic acid and *m*-chloroperbenzoic acid could be used to generate epoxides for HPLC selectivity or other purposes, the goal of the stress test in the current context is to expose drug substances only to the hydroperoxide oxidants that might realistically be encountered in solid dosage forms. In this way, structural enhancements to reactivity such as that suggested in Figure 3 are uniquely revealed and recognized.

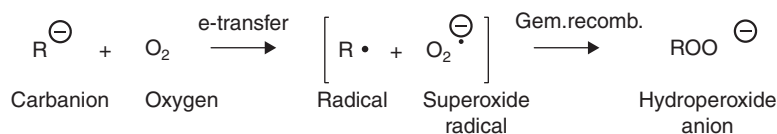


**Figure 3** Likely reaction mechanism of tetrazepam olefin bond with hydrogen peroxide.

**Ionization:**



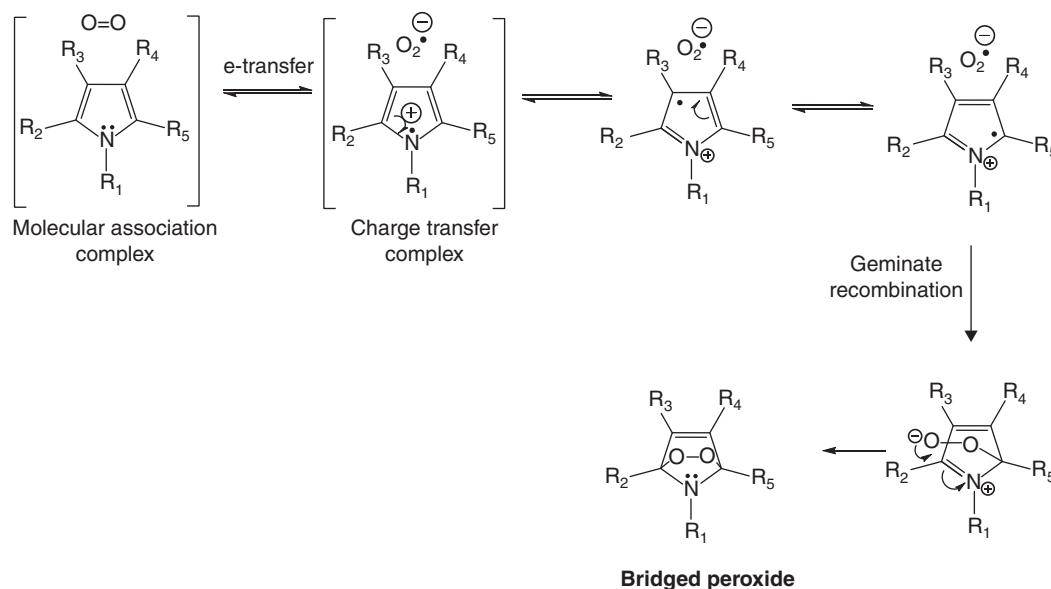
**Oxidation:**



**Figure 4** Ionization and oxidation steps in carbanion oxidation.

### Oxidation Mediated by SET to Dioxygen

While the oxidative mechanisms outlined in sections “Autoxidation” and “Oxidation by Organic Hydroperoxides and Hydroperoxide” above can explain a large number of oxidations, certain electron-rich moieties or compounds can undergo a “direct” SET to dioxygen. Two general groups of such compounds will be briefly considered here. The first is oxidation of carbanions, which has been generally treated or discussed in terms of base-catalyzed autoxidation (1). Compounds with weakly acidic hydrogen atoms, upon treatment with base, can yield carbanions which can react efficiently with dissolved oxygen. In fact, base treatment of such compounds in oxygen saturated solutions can proceed at very high reaction rates so as to be synthetically useful and has been reviewed (24). One view of the mechanism of the new C–O bond formation is by the mechanism shown in Figure 4 (25,26). After ionization, the carbanion undergoes SET to oxygen to give a carbon radical and superoxide radical. This “caged” radical pair is viewed to undergo geminate recombination (after one of the unpaired electrons undergoes a spin-flip) to form the hydroperoxide anion product. It is important to note that antioxidants such as BHT and BHA will have no effect in slowing the oxidation when geminate

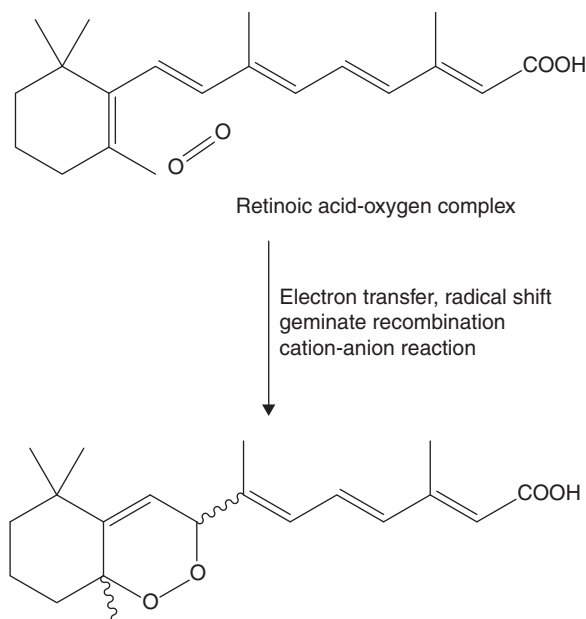


**Figure 5** Direct oxidation of substituted pyrrole adapted from Ref. 29; peroxide shown is one of numerous metastable products possible.

recombination dominates (Fig. 4) rather than escape of the radicals from the solvent cage, since there are no radical chains as described in section "Autoxidation." In a more pharmaceutically relevant context, Gu et al. (27) have reported such an oxidation mechanism for the autoxidation of ketarolac tromethamine in aqueous solution. Similarly, the oxidation of rofecoxib in higher pH solutions has been shown to proceed by this mechanism (28). Finally, a last example from this author's experience is certain amorphous salts of phenolate-containing drug candidates may have enough carbanion character (through contributing resonance structures) to be oxidized in the solid state as in Figure 4.

The second category of compounds, in which SET to oxygen can occur, is from electron rich, but formally neutral compounds. This has been demonstrated in compounds such as pyrroles (29),  $\alpha,\beta$ -unsaturated enamines (30), sterically strained cyclic olefins (31), and strained aromatic polycyclic compounds (32). The driving forces for SET are a high energy level of the highest occupied molecular orbital or a steric strain of the starting molecule. Complexation of oxygen by the electron-rich organic molecule has often been indicated as the first step of the mechanism. As such, a first-order kinetic curve in oxygen partial pressure is expected and is strong evidence of the direct oxidation mechanism, since autoxidation in Scheme 2 does not depend on oxygen pressure. Figure 5 shows the mechanism for a substituted pyrrole adapted from Beaver et al. (29). In the case of initially neutral compounds, the geminate recombination initially gives a cation and the hydroperoxide anion which may further react to give a bridged peroxide (Fig. 5). This type of pyrrole bridged peroxide oxidation product was recently implicated in the degradation profile of a pyrrole-containing pharmaceutical compound formulated in tablets and stored at 40/75% RH for 4 weeks (33). Although a mechanism was not explicitly offered, the current authors feel that a mechanism as in Figure 5 is very likely. A final example of this type of oxidation is retinoic acid, which can be oxidized by molecular oxygen without the need for radical initiators (34). The key evidence was the isolation of the product in Figure 6 that cannot be accounted for by the mechanism outlined in section "Autoxidation" but is predicted by the electron transfer mechanism in Figure 5.





**Figure 6** Retinoic acid and its cyclic peroxide supporting a direct electron transfer to oxygen mechanism.

### PRACTICAL TESTS AND CONSIDERATIONS FOR OXIDATIVE SUSCEPTIBILITY TESTING

The aim of oxidative susceptibility studies is to accelerate these “natural” oxidation processes described above, so that the intrinsic selectivity of the oxidants described in sections “Autoxidation,” “Oxidation by Organic Hydroperoxides and Hydroperoxide,” and “Oxidation Mediated by SET to Dioxygen” is maintained. Here “natural” means “occurring in solid dosage forms under normal long-term storage conditions and potentially under catalysis of common (tablet) excipient impurities. Since these studies are designed to mimic normal long-term oxidative processes, they can be thought of as “predictive” oxidative tests. Three goals of such testing can be identified:

1. To predict whether the substance is particularly sensitive to oxidation or not (semi-quantitative prediction). This enables comparisons to other drug substances so that formulation options/issues may be understood very early in development.
2. To discover specific oxidative degradation mechanisms, in order to prevent the degradation (e.g., Is oxidation peroxy radical mediated or SET to molecular oxygen?).
3. To produce the oxidative impurities profile that may be formed under accelerated and long-term storage conditions. This information facilitates development of appropriate stability indicating chromatographic methods.

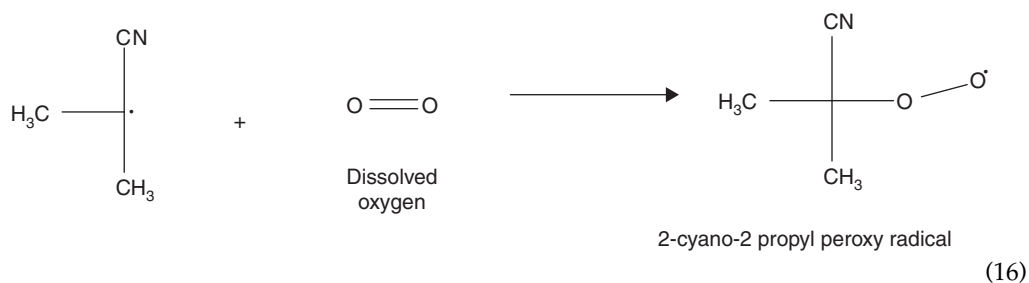
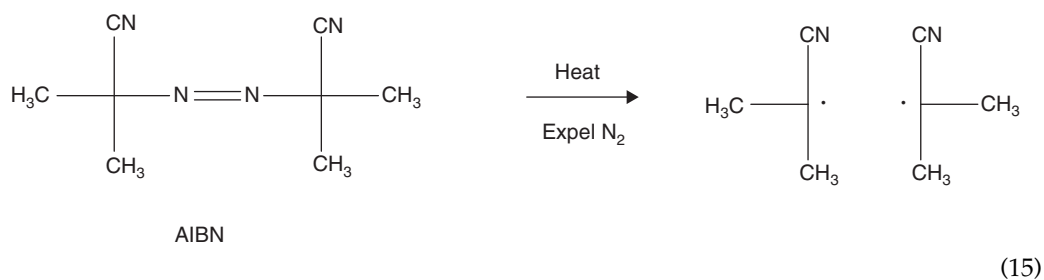
It is appropriate to mention here that other oxidative reagents and conditions may be appropriate for conducting *investigations* into oxidative degradation pathways. The purposes of such investigations include (a) the desire to make larger quantities of individual degradation products for structure elucidation or other purposes (via selectivity or faster kinetics) and (b) explorations of mechanistic aspects of the oxidative degradation pathways. Some suggested reagents and conditions for conducting oxidative investigations are shown in chapter 20, The remainder of this section will be organized similarly to section “Mechanistic Background for the Most

Common Oxidation Routes," focusing on practical, "predictive" tests and experimental aspects for determining the susceptibility of a compound to autoxidation, to oxidation by organic hydroperoxides and hydrogen peroxide, and to explore the potential for direct SET to oxygen. A key common theme is controlling experimental conditions to selectively produce the desired oxidants in the test solutions.

### Autoxidation

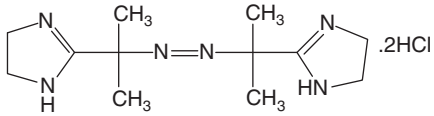
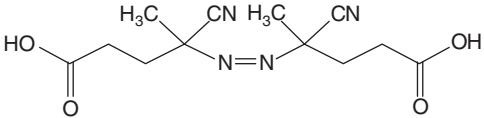
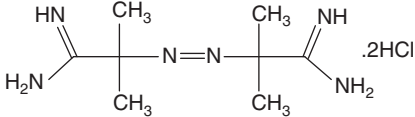
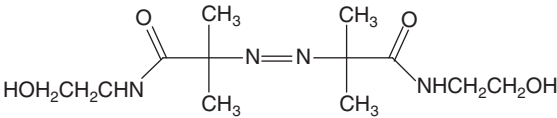
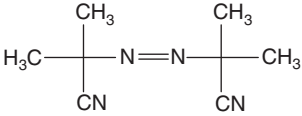
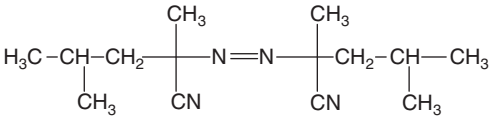
#### Use of Azo Compounds to Generate Peroxy Radicals

The autoxidation in Scheme 2 is selective due to the reactivity of peroxy radicals with low bond energy C–H bonds (or addition to olefin bonds) as described above. It is thus paramount to have a methodology which can generate exclusively peroxy radicals  $ROO\cdot$  in the subject test. Azo compounds (common examples in Fig. 7) including the popular azobisisobutyronitrile (AIBN), are well-known organic reagents capable of generating peroxy radicals by thermal decomposition in solution as shown in Eqs. (15) and (16) below for AIBN:



Upon heating, azo compounds expel nitrogen to generate carbon centered radicals which rapidly oxygenate at the diffusion controlled rate in oxygen saturated solutions to form 2-cyano-2 propylperoxy radical (in the case of AIBN). In the context of Scheme 2, this peroxy radical then "replaces"  $R_{\text{imp}}OO\cdot$  in Eq. (5a) and serves to initiate the oxidation of the substrate by its own peroxy radical [Scheme 2, Eqs. (6) and (7)]. Thus, when azo compounds are used in relatively low abundance with respect to an oxidizable substrate, Scheme 2 is a fair representation of the oxidation which takes place. In fact, much of the original work examining reaction rates of peroxy radicals with organic substrates was carried out in this limit. Azo compounds were typically used at only a few mole percent relative to the oxidizable substrates being studied (35,36), and substrate concentrations were high (often neat liquids). In this limit, the azo compound-derived peroxy radicals readily abstract hydrogen atoms from substrate and are converted to stable hydroperoxides.

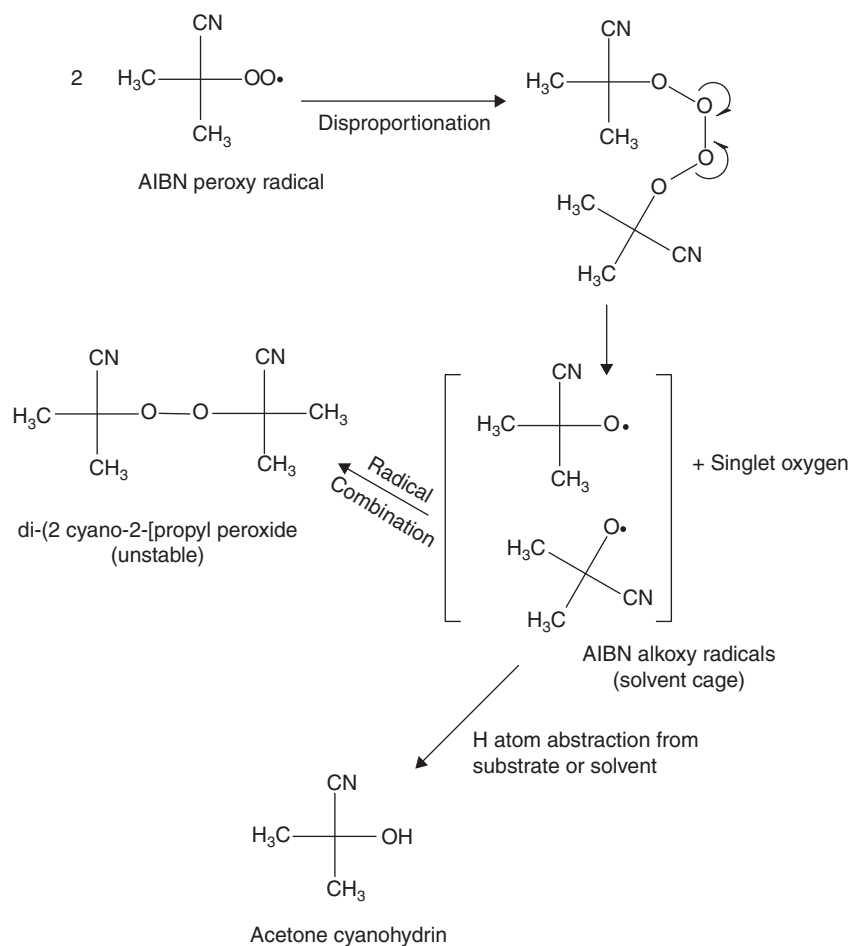
However, the use of azo compounds by the pharmaceutical industry for the subject oxidative test has evolved to a different limit in which the azo compound "initiator" is often used in significant molar excess compared to the dilute drug substance, and substrates are much more dilute. This can have a negative impact on the selectivity of the oxidative test, as we shall

Chemical name	Structural formula	10 hour half-life	Solubility
2,2'-Azobis( <i>N,N'</i> -dimethylenisobutyramidine) dihydrochloride		44C in water	35.2 mg/mL in water
4,4'-Azobis(4-cyanopentanoic acid), ACVA		69C in water	1 mg/mL in water
2,2'-Azobis(2-amidino-propane)dihydrochloride, AAPH		56C in water	23.2 mg/mL in water
2,2'-Azobis[2-methyl-N-(2-hydroxyethyl) propionamide]		86C in water	2.4 mg/mL in water
2,2'-Azobisisobutyronitrile, AIBN, VAZO64		65C in toluene	7.5 mg/mL in methanol
2,2'-Azobis(2,4-dimethylvaleronitrile, VAZO52, AMVN		51C in toluene	22 mg/mL in methanol

**Figure 7** Common azo compounds used in oxidative stress testing shown from top (least water soluble) to bottom (most water soluble).

see shortly. At this point, a historical overview of the azo compound to substrate mole ratio used in pharmaceutical applications is warranted. The first use of azo compounds to examine autoxidation of pharmaceutical compounds in solution was Oyler in 1991 (37), Boccardi in 1992 (23), and Boccardi in 1994 (38). In these cases, AIBN was in molar excess compared to the drug substance, the AIBN/drug molar ratio being about 2:1 (60 mM AIBN/35 mM drug) and 10:1 (170 mM AIBN/15 mM drug) for Boccardi and Oyler, respectively. These studies, in particular Boccardi's detailed work on tetrazepam (5,38), established the potential of azo compound "initiation" in solution to mimic autoxidation in solid dosage forms and started a more general usage of azo compounds for examining the oxidation potential of drug substances. By 2003, Alsante et al. (39) surveyed the pharmaceutical industry in regard to forced stress testing practices and found that azo compound initiators were commonly used with AIBN in water-acetonitrile-based solvent systems being typical. Currently, drug substances are being examined more and more early in development, when only mg quantities may be available. As a result, common drug concentrations for the subject test in a current pharmaceutical context have significantly diminished, while the azo "initiator" to drug molar ratio has remained near 10 to 1 (typical concentrations currently might be about ~5 mM azo compound and ~0.5–1 mM drug substance).

This sets the stage for potential selectivity problems in the current pharmaceutical context of the azo compound experiment. Let us assume based on bond energies that peroxy radical reaction rates with common a solvent such as acetonitrile (or methanol) are negligible (15).



**Figure 8** Disproportionation reaction of AIBN peroxy radical in the absence of any oxidizable substrate or solvent, yielding 2-cyano-2 propoxy radical.

Then, at large azo compound to substrate molar ratios, with dilute substrates that are potentially not reactive or only moderately reactive toward peroxy radical, some disproportionation of the azo peroxy radicals can be expected since rate constants for peroxy radical disproportionation are large compared to any C–H bond abstraction rates [on the order of  $2k_t \sim 10^3\text{--}10^7 \text{ M}^{-1} \text{ s}^{-1}$  (10,18)]. This disproportionation reaction is shown specifically for AIBN in Figure 8. The selectivity issue is that all azo compound peroxy radicals are *tertiary* peroxy radicals, and as such, disproportionation of these peroxy radicals must generate alkoxy radicals,  $\text{RO}\cdot$ . The bond energies of  $\text{RO-H}$  species are near 105 kcal/mole (15) as compared to that of 89 kcal/mole for  $\text{ROO-H}$  bonds as discussed previously. Thus, alkoxy radicals are not selective in that they will react with much stronger C–H bonds than peroxy radicals. This would signal “incorrect” (i.e., nonpredictive) oxidative degradation profiles. Further, even if the alkoxy radicals in Figure 8 reacted with the “appropriate” C–H bond (i.e., a C–H bond that peroxy radicals can also react with) that reaction rate will typically be  $10^4\text{--}10^6$ -fold larger than H-atom abstractions by peroxy radicals (36). For example, the ratio of *tert*-butoxy radical to *tert*-butyl- peroxy radical H-atom abstraction rates for cumene, tetralin, tetrahydrofuran, and toluene are  $\sim 10^6$ ,  $\sim 4 \times 10^5$ ,  $\sim 1.5 \times 10^5$ , and  $\sim 4 \times 10^5$ , respectively (36). Thus, in addition to the selectivity issue, relatively low levels of

“strong” alkoxy radicals (2-cyano-2 propoxy radicals in Fig. 8) could also “signal” significant peroxy radical reactivity of the dilute drug substance when in fact, there was very little reactivity. Either case is a significant issue for the subject test.

#### *Choice of Solvent Composition to Minimize Alkoxy Radical Activity*

Several examples of solvent effects for azo compound oxidation related to Figure 8 have in fact been recently reported in the pharmaceutical context and will be briefly described here. Using 5mM AIBN or ACVA in 100% acetonitrile with 0.5mM cumene (0.1 mg/ml) as a substrate (a model for a dilute drug substance), Nelson et al. (40) found that the total oxidation products of cumene decreased 5-fold (for ACVA) and ~10-fold (for AIBN) upon addition of only ~2–5% by volume methanol to the acetonitrile test solution. The authors argued that the actual “oxidant” in the absence of methanol solvent was in fact the AIBN-related alkoxy radical in Figure 8, and that the addition of even small amounts of methanol served to “quench” the 2-cyano-2 propoxy radical (by rapid donation of a H atom by the methanol). The expected methanol oxidation products were detected (i.e., formic acid and formaldehyde). As further supporting evidence, the authors noted that in the case of cumene oxidation, the cumene alkoxy radical can serve as a type of “internal clock” that is sensitive to the effective H atom donation ability of the solvent. The tertiary cumene alkoxy radical (formed by disproportionation) undergoes an internal  $\beta$ -scission rearrangement to form acetophenone. This rapid internal rearrangement competes with H-atom abstraction from solvent to yield 2-phenyl-2-propanol. The acetophenone/2-phenyl-2-propanol product ratio was found to be steadily reduced by 2-fold by addition of only 1–3 % methanol (vol%) to the acetonitrile. This was interpreted as ca. 50-fold higher H-atom donation rate by methanol to the cumene alkoxy radical (as compared to acetonitrile). This supported the authors’ proposed rapid H-atom donation rate of methanol (compared to acetonitrile) to the 2-cyano-2 propoxy radical.

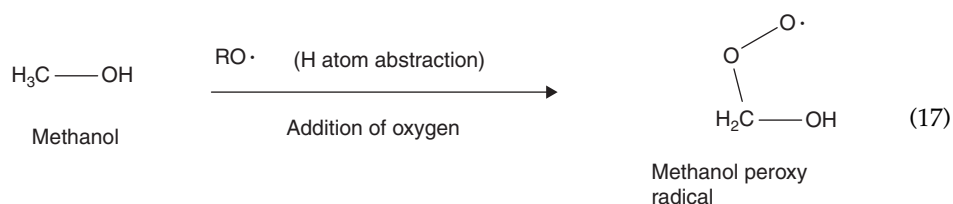
Watkins et al. (41) have also reported similar results in which the addition of low levels of methanol to acetonitrile/water AIBN and ACVA initiated oxidation experiments eliminated significant (3–10% levels) oxidative degradates which would have otherwise been presumed to be “peroxy radical” mediated. These authors isolated the degradation products and showed by NMR structural elucidation that, in fact, the degradation was from the addition of the 2-cyano-2 propoxy radical to an aromatic ring system. This result unequivocally demonstrates the alkoxy radical activity shown in Figure 8 in acetonitrile-water cosolvent systems under current pharmaceutical azo compound stress testing conditions (i.e., in the presence of acetonitrile without the presence of methanol). Methanol H-atom donation to the alkoxy radical was again rationalized as the mechanism by which methanol removed this unwanted reactivity. Once the *alkoxy* radical activity was quenched, there was no observed degradation of the drug from the remaining *peroxy* radicals generated by either AIBN or ACVA (41).

Given that this azo-derived alkoxy radical activity may be somewhat surprising to practitioners in the field, and that it can significantly undermine the subject oxidative test result conclusions for substrates unreactive toward peroxy radicals, several other considerations regarding the reaction shown in Figure 8 should be detailed. High yields of acetone cyanohydrin, derived from H-atom abstraction of the 2-cyano-2 propoxy radical as shown in Figure 8, have been shown for the AIBN-initiated oxidation of neat benzene (85% yield) and neat xylene (20% yield) in oxygen-saturated solutions using 0.6M AIBN with a reaction temperature of 50°C (42). These data clearly show efficient disproportionation, as shown in Figure 8, even in the presence of *molar* concentrations of marginally reactive substrates (benzene and xylene).

Another point to consider in understanding the “reality” of the reaction in Figure 8 is the relative amount of AIBN peroxy radicals formed, compared to the dilute drug substance oxidized. Using the known Arrhenius parameters for AIBN decomposition, and a mean oxygenation efficiency of 50%, Boccardi (5) estimated that after 2 days at 40°C, the yield of the 2-cyano-2 propoxy radical will be 5% relative to the starting AIBN concentration. Taking this 5% conversion value and the typical 5mM AIBN concentrations used, over a 2 days’ test period 0.25mM 2-cyano-2 propoxy radical will be generated. This can be compared to the amount of drug

oxidized in a “typical” case, for example, assume 5% degradation of the initial 0.5 mM drug substance, and thus, 0.025 mM drug is degraded. Over the 2 days’ test period there is 10X more 2 cyano 2 propyl peroxy radical generated than drug oxidized, and the disproportionation in Figure 8 is inevitable if the solvent is also unreactive with peroxy radical.

It is possible that some of the azo compounds in Figure 7 may have different effective yields of their own alkoxy radicals, or that the alkoxy radical yields have some solvent dependence. However, a general solution to the azo compound disproportionation problem is to recommend that at least 10% methanol should be added to acetonitrile–water solvent systems to quench any potential azo compound alkoxy radical activity. Alternatively, methanol–water and or ethanol–water cosolvent systems (without any acetonitrile) will offer the same alkoxy radical quenching advantages and should not otherwise significantly alter overall peroxy radical-mediated oxidative yields or degradation profiles (38). However, if hydrolytically sensitive intermediates (such as epoxides) are generated, then use of high methanol- or ethanol-based solvents may give different degrade profiles due to solvolysis. Note that the H-atom donation by methanol to an alkoxy radical produces a methanol radical that will oxygenate to form a methanol peroxy radical as shown in Eq. (17), which should have the appropriate selective reactivity for the subject test. For completeness, it should be pointed out that this methanol oxidation will lead to formaldehyde and formic acid (at a few  $\mu\text{M}$ ) in the sample (41):



#### Oxygenation Requirements

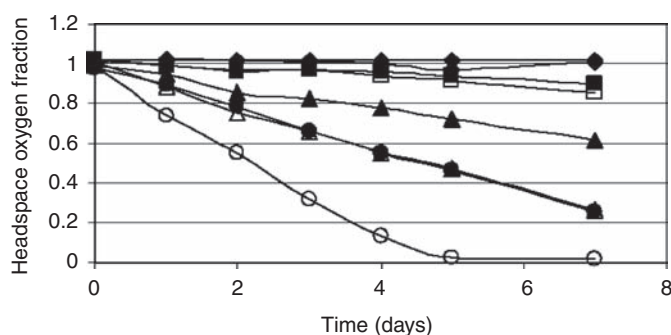
As described in section “Autoxidation”, expressions for the rate of autoxidation in solution are proportional to the product of the substrate concentration and the rate constant of the peroxy radical hydrogen atom abstraction, which is relatively slow. Given the oxygen solubility in liquids in equilibrium with ambient oxygen and the rapid rate of Eq. (6) in Scheme 2, there is typically no oxidation rate dependence on the dissolved oxygen concentration (18,19). Thus, the typical experimental conditions for the subject autoxidation test in regard to oxygenation has been to use unstirred solutions, in capped flasks with roughly an equivalent solution volume of ambient atmosphere over the solution. Given a range of potential initiator concentrations which could be used and widely varying substrate reactivities, it is reasonable to consider if enough dissolved oxygen remains in solution during an autoxidation test as just described. Some investigators have used enriched or pressurized oxygen atmospheres to ensure a maximal degradation rate in azo-initiated oxidation experiments. Many researchers feel some reticence in enriching oxygen levels in combination with the potentially explosive azonitriles. Fortunately, some measurements have recently been made which are informative. Nelson et al. (43) measured oxygen remaining in the headspace over equal volumes of AIBN and ACVA in solution at 1, 5, 25, and 50 mM in the absence of substrate at 40°C. Figure 9 shows the AIBN data for oxygen levels remaining in the headspace over 7 days; both with and without stirring of the solutions. After 2 days, there is little depletion observed for the 1 and 5 mM AIBN levels both with and without stirring; even the 25 mM AIBN cases are only ca. 20% depleted, and only marginal differences between stirred and unstirred. The presence of substrate will increase the oxygen consumption and needs to be considered briefly. Assuming drug substance at 0.5 mg/mL or 1 mM, and again using the estimated 5% AIBN yield over 2 days (5), we can estimate that the oxygen consumption in Figure 9 corresponds to 0.25 mM AIBN peroxy radical formed. This corresponds to 25% of the drug present. Thus, even if *all* the drug were consumed by subsequent

propagation steps, the total oxygen consumed would be 4X greater- or about similar to the 25 mM AIBN data in Figure 9 (around 20% depleted). Thus, the data support the current general practice of working with ca. 5 mM AIBN levels in unstirred solutions. The AVCA data (43) show about 2-fold more oxygen consumption, and similarly supports 5 mM initiator concentrations (and low mM drug concentrations) in unstirred flasks.

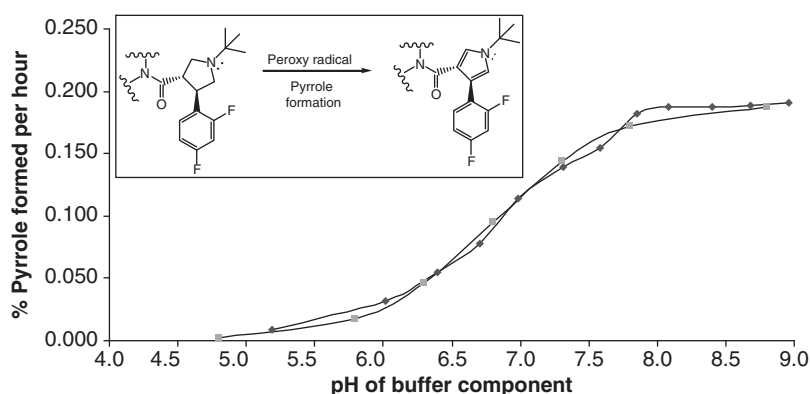
Figure 9 does highlight that use of significantly higher initiator and drug concentrations (50 mM) will likely lead to substantial depletion of oxygen in the bulk solution. While it is by no means clear that this would adversely affect the general results of the subject test, it is important nonetheless to be aware of the resulting nonlinear kinetics and the potential for alterations in degradation profiles.

#### Solution pH and Temperature

Generally, control of the solution pH during azo compound-initiated oxidation has not been highlighted in the literature. However, given the abundance of amine groups in pharmaceutical compounds, it is plausible that C-H bonds adjacent to amine groups could have a susceptibility to H-atom abstraction by peroxy radicals which is dependent on the protonation state of the nitrogen atom lone pair of electrons. Figure 10 shows this effect quite elegantly for a peroxy radical-mediated oxidation of a pyrrolidine ring to an aromatized pyrrole ring. There



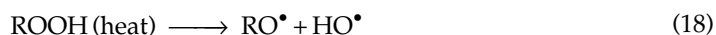
**Figure 9** Headspace oxygen concentration as a function of time for 50% acetonitrile solutions containing (◆) 1 mM AIBN, (◇) 1 mM AIBN with agitation, (■) 5 mM AIBN, (□) 5 mM AIBN with agitation, (▲) 25 mM AIBN, (△) 25 mM AIBN with agitation, (●) 50 mM AIBN, and (○) 50 mM AIBN with agitation. Solutions were stored at 40°C and either stirred or left static in between measurements. Oxygen levels are normalized to the results measured each day for a 50% acetonitrile control sample.



**Figure 10** pH dependence of the AIBN oxidation of a drug molecule to form a pyrrole moiety. Gray squares (calculated pH curve, described in the text), and black diamonds are experimental values.

is a net mass loss of 4 amu from the parent compound; the conversion can be envisioned as resulting from two reactions with peroxy radical and two subsequent eliminations. Figure 10 shows the relative rate of the pyrrole formation (as a % initial drug peak area) as a function of the pH of the 50% aqueous (20 mM phosphate buffer) portion of the solvent; the other 50% being methanol. The black data points and solid line are the data; the gray data points and curve are to guide the eye and are a simple equilibrium pH calculation of normalized concentration of the deprotonated form of an amine group with a  $pK_a$  of 6.8 in the mixed solvent system. The data clearly show that the overall pyrrole oxidation rate is controlled by the protonation state of the pyrrolidine ring nitrogen atom. The pyrrole oxidation rate increases 20-fold from an apparent pH 5.0 to 8.0. Figure 10 highlights that if this compound were available as an HCl salt versus a free base, and tested at 0.5 mM in an unbuffered acetonitrile/methanol cosolvent system, two considerably different pyrrole oxidation yields would likely be obtained. These data warrant considering pH control of azonitrile-initiated oxidations if the substrate has amine (or other) moieties with  $pK$ 's in the pH range of ~ 4 to ~ 9 in the mixed solvent system.

The final experimental variable to be considered is the temperature of the oxidation experiment. The temperature is a balance between the need for the thermal decomposition of the initiator (Eq. (15)) and trying to minimize thermal degradation of hydroperoxides or peroxides as shown in Eqs. (18) and (19):



Generation of these strong alkoxy and hydroxyl radicals would degrade the selectivity of the subject test as described previously in detail for  $\text{RO}^\bullet$  radicals. Hydroxy radical is similar in that it is a much stronger radical than peroxy radical. Given the estimation described here of 5% yield of AIBN peroxy radicals over 2 days at 40°C, and the typical drug concentrations used, 40°C is recommended as the base case temperature to carry out the subject test. Higher temperatures could be used, but the contribution of total oxidative products by oxidation via Eqs. (18) and (19) would be increasingly difficult to determine. This could mask overall reactivity of the substrate with peroxy radical, which is one primary outcome of the experiment. Higher temperatures also reduce dissolved oxygen concentrations. Table 1 summarizes the azo compound oxidative test conditions recommended here.

**Table 1** Recommended Conditions for the Oxidative Susceptibility Tests

Test	Desired Oxidation Mechanism	Temperature	Concentrations of Oxidant	Concentrations of Substrate	Solvent Composition	pH Control?	Duration
Hydrogen peroxide	2 electron oxidation	≤RT	≤0.3% by volume	0.1–1 mM	water/ACN with methanol	Yes if amines	≤24 hours
AIBN	Peroxy radical oxidation	40°C	~5 mM	0.1–1 mM	≥10% methanol, ACN, water	Yes if amines	≤48 hours
Transition metals	Electron transfer	40°C	~1 mM	0.1–1 mM	ACN/water (low methanol)	Neutral pH	≤72 hours



### Alternative Peroxy Radical-Based Oxidative Stressing System

The azo compounds in Figure 7 are toxic and can explode under certain conditions. For these reasons, some laboratories have difficulty obtaining azo compounds. There has been another methodology reported for generating a peroxy radical-based oxidative stressing system without the use of azo compounds (44). This oxidative system leverages Scheme 1. Tween 80 is used at 10% by weight in aqueous solution to provide high levels of ROOH and Fe(III) is added at 10 mM. Although peroxy radicals are formed along with alkoxy radicals (Scheme 1), the 100 mg/mL levels of oxidizable Tween 80 reacts with those alkoxy radicals prior to encountering sub-millimolar concentration substrates thus preserving the peroxy radical activity. General oxidizability rankings with this system appear consistent with AIBN-initiated systems (44). One significant disadvantage of the Tween 80/Fe(III)-based system is that Tween 80 is not amenable to subsequent LC-MS analysis of the oxidative degradates formed.

### Oxidation by Organic Hydroperoxides or Hydrogen Peroxide

Section "Oxidation by Organic Hydroperoxides and Hydroperoxide" highlighted the potential reactions of hydrogen peroxide or organic hydroperoxides ROOH with drug molecules. The intact hydroperoxide may act as either an electrophile or a nucleophile. Experimental conditions must be optimized to ensure that this two electron or paired electron reactivity is the only significant oxidative reaction possible during the test measurement. Thus, critical experimental parameters are the test temperature, the solvent composition, and the concentration of hydrogen peroxide. These will be considered in sections "Hydrogen Peroxide Level and Temperature" and "Water Cosolvent System". In section "pH of Cosolvent System", we will discuss the effects of the test solution pH in the case where drug substance amine group reactivity is being explored.

#### Hydrogen Peroxide Level and Temperature

The two electron reactions being probed with the hydrogen peroxide test are relatively rapid at room temperature, and thus there is little need to elevate reaction temperature beyond ambient. Increased reaction temperatures come with higher risk associated with creation of additional undesired hydroxyl radical oxidants as shown in Eq. (20). The peroxide bond in hydrogen peroxide has a bond energy of about 213 kJ/mol (50 kcal/mol) (45). Homolytic decomposition of hydrogen peroxide will increase in rate as the temperature is raised:



Hydroxyl radicals are strong, nonselective oxidants as described above, and can rapidly react with drug substances in solution and confound the normal peroxide (i.e., paired electron) reactivity being examined in the subject test.

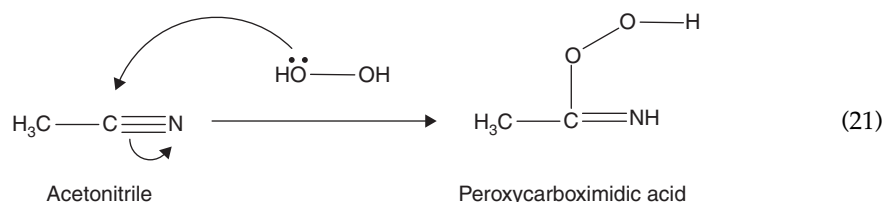
Hydrogen peroxide concentrations do not need to be more than 0.3% (which corresponds to ~90 mM) and can often be 10-fold lower (~9 mM) as the paired electron reaction rates are generally much faster than peroxy radical reactions. Drug concentrations are convenient to use at submillimolar concentrations rationalized in section "Autoxidation". A stress period of 24 hours should be adequate time to show a reaction with hydrogen peroxide (or, as important, to show the lack of a reaction).

#### Water Cosolvent System

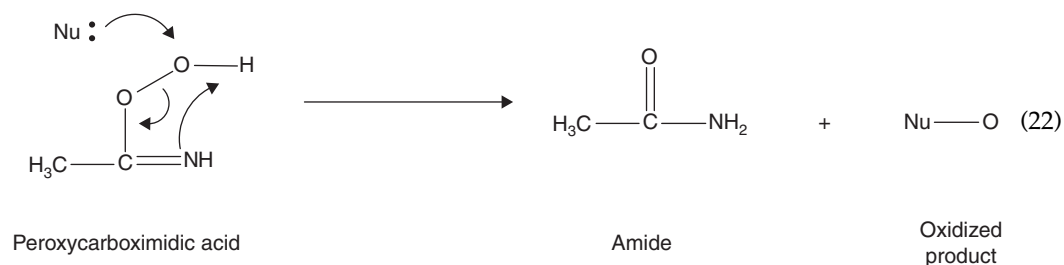
It is very important that a cosolvent be used in addition to water. Methanol and acetonitrile will serve to "quench" any low-level hydroxyl radical activity produced by Eq. (20) (even at room temperature) by donation of a hydrogen atom to the hydroxyl radical. In this regard, it has been this author's practical experience that methanol appears to be a better H-atom donor to hydroxyl

radical than acetonitrile, similar to the conclusion reached by Nelson et al. (41) regarding methanol being a better H-atom donor to the 2-cyano-2 propoxy radical compared to acetonitrile. Thus at least 20% methanol is recommended as a cosolvent (remaining being water, acetonitrile, or methanol). It should be recognized that cosolvent quenching of hydroxyl radicals thus leads to low levels of solvent peroxy radicals (as shown in Eq. (17) for H-atom donation of methanol). Therefore, minor peroxy radical degradate peaks may also form over the 24-hour hydrogen peroxide test duration. Any hydroperoxide paired electron reactivity should be much larger in comparison.

There is, however, a clear preference for methanol as a cosolvent if a higher pH range of the hydrogen peroxide reaction with the drug substance needs to be explored (as described in the next section). At higher pH in a water–acetonitrile cosolvent system, acetonitrile has the liability of being able to react with hydrogen peroxide to form peroxyacetic acid (46,47) as shown in Eq. (21):



Peroxyacetic acid is an unstable oxidizing species; it is even more reactive than hydrogen peroxide as it can undergo reaction with even weaker nucleophiles (Nu in Eq. (22)) due to the more favorable leaving group compared to hydrogen peroxide:



Since a drug substance will not encounter peroxyacetic acid in a pharmaceutical dosage form, any oxidized product formed as in Eq. (22) is an unnatural and undesired oxidation event for this test. Methanol/water or ethanol/water are more appropriate and common solvent systems which avoid this problem and provide excellent solubility for most drug substances.

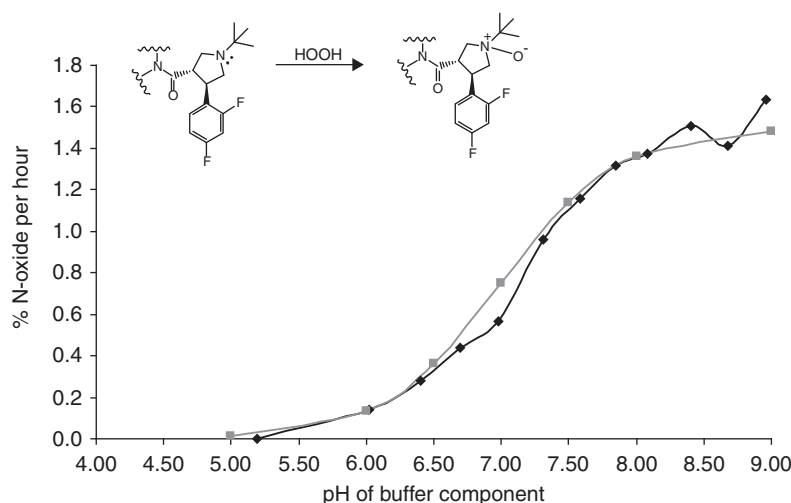
#### *pH of Cosolvent System*

The reaction of amines with hydrogen peroxide shown in Eqs. (11) and (12) will slow dramatically when the nitrogen lone pair of electrons is protonated. Thus, some attention should be given to the cosolvent apparent pH during the hydrogen peroxide test. Figure 11 shows the typical trends one can expect for a tertiary amine. The cosolvent system is 50% methanol, 50% phosphate buffer adjusted to the pH values shown on the *x* axis. The hydrogen peroxide concentration is 0.3%, the drug is present at 0.1 mg/mL and the stress temperature is ambient. The *y* axis gives the *N*-oxide formation rate observed (determined from the slope to a

linear fit of four data points over the first 6 hours) in % total drug present converted to the *N*-oxide (per hour). The black data points show the actual data, while the gray data points are to guide the eye and derive from a simple calculation of the protonated/deprotonated amine nitrogen ratio based on an apparent  $pK_a$  of 7.0 in the mixed solvent system. Thus, the protonation state of the amine nitrogen is controlling the reaction rate as expected. The simplest recommendation would be to carry out the hydrogen peroxide test with the pH controlled near the  $pK_a$  of the amine in the cosolvent system if amine groups are present. In Figure 11, this gives ~10% formation of tertiary *N*-oxides with overnight stressing. Note that if the reaction had been inadvertently carried out at an apparent pH near 4, the entire *N*-oxide formation might have been missed as a potential oxidation route. Table 1 summarizes the recommended test conditions.

### Oxidation Mediated by SET to Dioxygen

In studying the reactivity of a new drug substance, it is obviously interesting to ascertain whether it can undergo SET to molecular oxygen at an appreciable rate, but this is often not a simple task. An obvious step is to start by monitoring an oxygen-saturated solution of the test compound with no azonitrile initiator or hydrogen peroxide added. However, bearing in mind the trace levels of initiators acting in Scheme 1 and their efficacy at very low concentrations, it is practically impossible to exclude them totally. Nevertheless, the first clue of an oxidation mediated by direct electron transfer to oxygen is oxidative degradation in experiments performed with particular care using very pure reagents and very clean apparatus. Often there appears to be no significant induction period to the oxidation. The most convincing evidence, as discussed in section "Mechanistic Background for the Most Common Oxidation Routes", is that the oxidation will typically show a first-order dependence on the dissolved oxygen concentration even at ambient (saturated) dissolved oxygen levels. However, there are some experimental approaches to be considered in the context of gauging the susceptibility of a compound to undergo SET to oxygen. In the current context, we will consider oxidation of the substrate by Fe(III) and Cu(II) transition metal complexes as well as application of electrochemical methods, and will discuss the former first.



**Figure 11** Formation of tertiary *N*-oxide as a function of pH of the aqueous phase of the methanol-phosphate buffer cosolvent system. Gray squares are calculated pH curve, and black diamonds are experimental data.

*Use of Fe(III) and Cu(II) Transition Metal Ion Complexes*

Transition metal complexes may catalyze oxidation in a number of ways. A brief discussion is worthwhile to frame any relevance of such tests to the potential for SET to dioxygen. Four very general modes of metal ion catalysis of oxidations can be considered (for a review, see Ref. 10):

1. metal ion oxidation–reduction reactions with hydroperoxides as shown in Scheme 1;
2. metal ion complex activation of molecular oxygen;
3. direct reaction of metal with substrate- “outer sphere” or electron transfer; and
4. direct reaction of metal with substrate- “inner sphere” or ligand transfer

In the context of possibly probing a drug candidate’s propensity for SET to dioxygen then, we are primarily interested in mechanism (*iii*) above, in which the coordination sphere of the metal ion remains intact. However, it is not straightforward to distinguish this from mechanism (*iv*) which involves a coordination of the metal ion with the substrate followed by electron transfer (10). The “ease” of the electron transfer in both mechanisms (*iii*) and (*iv*) will generally be related to the ionization potentials of the substrates. In either case, in our view transition metal complexes of iron(III) and copper(II) are appropriately discriminating agents for this type of test as their redox potentials in aqueous solution are not excessively positive, being near 0.77 and 0.15 V (versus NHE), respectively. For example, Harmon et. al (9) found in control experiments that 10 mM Fe(III) chloride in water/acetonitrile mixtures did not give significant oxidation of any of the 18 compounds being studied in that work. In contrast, Boccardi (23) found that 1.5 mM Fe(III) chloride and 1.5 mM Cu(II) sulfate (in acetonitrile) oxidized 40–50% of the initial tetrazepam present. These data indicate the selectivity of the subject iron(III)- and Cu(II)-based test.

Iron(III) and Cu(II) do not typically participate in mechanism (*ii*) as long as no source of reducing equivalents are present and mechanism (*i*) above is obviated by the use of clean simple solutions of the drug being studied. Thus oxidation observed during this metal ion test can generally be interpreted as due to electron transfer mechanisms (*iii*) or (*iv*) above. While neither process may predict SET to oxygen, this test does provide a simple means to get a measure of the electron transfer potential of the drug substance. Typical solvent test conditions would be acetonitrile/water solvent mixtures; however it is recommended that a small amount of methanol be added to reduce any alkoxy radical activity that might be generated. Reaction temperature is recommended  $\leq 40^\circ\text{C}$ , and metal ion complex concentrations at ca. 10–100 mol% relative to the drug substance. Drug substance concentrations might range from 0.1 to 1 mM depending on drug substance availability, but the concentration is not critical. Any number of salts and complexes of iron(III) and copper(II) can be used (5). Table 1 summarizes the test conditions.

*Cyclic Voltammetry: Electron Transfer from Substrate to an Electrode*

Cyclic voltammetry (CV) has been applied to the determination of the potentials at which drug substances in solution can be reversibly oxidized at an electrode surface (7,8). It is for this reason we chose to discuss the CV methodology in the context of methods which might shed light on substrates prone to electron transfer to dioxygen. However, the main focus of the CV work has been to examine the potential of the methodology as a general oxidative screen; similar to azo compound initiated oxidation discussed here.

For example, Lombardo and Campos (7) describe, in parallel with an HT protocol using a radical initiator (ACVA) at  $60^\circ\text{C}$ , a HPLC-electrochemical method using an array of 12 electrodes at potentials between  $-0.2$  and  $1.2\text{ V}$  (with reference to a Pd electrode). Compounds were examined and were ranked in six classes in decreasing order of oxidation sensitivity based on the observed oxidation potential. These results were compared to the % compound remaining in the ACVA initiated oxidation. While many compounds were ranked as stable by both techniques, there was an “orthogonal” aspect to data for some compounds; where for example, compounds that ranked as the most unstable by CV oxidation potential ranged from 0% to 83%

consumed in the AVCA initiated oxidation test (7). Gamache and co-workers (8) showed data obtained by using the same CV apparatus of Lombardo and Campos, on 22 known drug substances and antioxidants. The authors ranked substances as unstable or stable based on oxidation potential.

In our opinion, the main drawbacks of the application of CV electrochemical methods as a *general* oxidative screening method is the fact that anodic oxidation is an electron-transfer reaction which is not that common an oxidative pathway in solid dosage forms as discussed above. A compound could in principle be resistant to oxidation by electron transfer to dioxygen, but very prone to reaction with peroxy radical (and vice versa). In fact, it is our naïve hope that this is the nature of some aspects of the “orthogonality” noted by Lombardo and Campos. However, at this time that correlation has not been demonstrated, but efforts spent in development of CV methods focused on predicting/clarifying/correlating the potential for direct electron transfer to dioxygen would be of significant value. In this sense, our view is similar to some perspectives of Waterman and co-workers (3), who also described the use of CV to study the oxidation sensitivity of known drugs and concluded that CV is more appropriate for detailed mechanistic studies than for fast general oxidative screening.

### SUMMARY AND GENERAL STRATEGY OF OXIDATIVE SUSCEPTIBILITY TESTING

Section “Practical Tests and Considerations for Oxidative Susceptibility Testing” began by highlighting primary goals of oxidative susceptibility testing- to understand a compound’s liability to oxidation early on, so as to inform formulation efforts and optimization strategies from the start. In order for this approach to work, the solution-based oxidative stress must generate only the oxidizing agents found in solid dosage forms. In that regard, peroxy radicals and organic hydroperoxides have been put forth here as the most common oxidizing agents. The careful choice of experimental conditions described here (summarized in Table 1) can reduce the effects of undesirable oxidants generated in the AIBN and the hydrogen peroxide tests, and thus provide the clearest view of the true oxidation potential.

In this context, it is useful to consider the “interpretation” of the levels of degradation one might encounter in these oxidative tests. If Table 1 is followed as recommended, it is this author’s experience that for the AIBN test in methanol containing solvents, a good number of drug substances will degrade very little if alkoxy radical activity has been eliminated. It is not uncommon to see only 0–2% drug lost during an AIBN test (41–43) and as such these compounds would be classed as oxidatively stable toward peroxy radical. Similarly many molecules (except tertiary amine containing drugs) also give 0–2% loss in the hydrogen peroxide test carried out as in Table 1. Molecules giving these types of low percentage assay loss in the AIBN and hydrogen peroxide test results can be expected not to have any issues with oxidation in solid dosage forms regardless of formulation strategy in this author’s experience. This, in fact, is the one of the best “predictions” that oxidative susceptibility testing can provide—the *lack* of sensitivity. On the other hand, a result of greater than 20% drug loss in the AIBN test signals the compound is susceptible to peroxy radical oxidation and would require careful selection of excipients and, potentially, the use of antioxidants. The hydrogen peroxide test delivers high concentrations of oxidant and should show large % conversions (5–100%) of the drug if such a two electron ionic reactivity is present. If found, it predicts some sensitivity to peroxide containing or peroxide generating excipients; but fortunately, the reaction of drug molecule with a ROOH group in this way is stoichiometric and the drug is often in large excess compared to the trace ROOH levels.

Transition metal ions such as iron(III) and copper(II) may allow detection of substances with a low redox potential. This may be useful in the context of understanding the potential for oxidation by electron transfer to oxygen. If the test compound is sensitive to a number of different oxidants or catalysts, such as AIBN, hydrogen peroxide and iron(III), it is advisable to consider the substance as potentially very sensitive to oxidation. In this case, it is wise to consider preformulation efforts investigating the use of antioxidants or special protecting conditions.

A second tier of tests should be designed for compounds already detected as sensitive. The goals of the second tier are the isolation of impurities and a more detailed investigation of the degradation mechanism. It is not possible to propose general protocols, as in this case the chemistry of the substance must be fully considered. In this phase, we can study, by comparison with known examples and data, solvent effect, pH effect, detection of hydroperoxides, or the use of singlet oxygen or oxidants that are more selective for the structural class of interest. Once the degradation profile is ascertained, more in-depth studies can be recommended to further guide preformulation activities.

### FUTURE DIRECTIONS

Oxidation problems in solid dosage forms are most often caused by peroxy radical and as such, the AIBN test (or other similar compounds in Fig. 7) is likely the single most important test in determining the likelihood of potential oxidative issues in a solid dosage form. Table 1 now provides a means to reduce "false positives" in this test, in particular by careful selection of solvent composition. This improvement should allow for better semi-quantitative correlations to be developed between AIBN test "% claim lost" values and formulation routes needed (if any) to stabilize the drug substance. Published examples of data sets comparing long-term (oxidative) stability performance in pharmaceutical dosage forms and AIBN test results would be beneficial in this regard.

Another useful area of endeavor would be to document a methodology which would allow a researcher to determine if their azonitrile system was "working properly." That is, is the azonitrile compound liberating the right amount of carbon centered radicals? Is enough oxygen reacting with those radicals to produce the appropriate amount of azonitrile-derived peroxy radical? A possible solution to this problem is to monitor the quantity of hydroperoxides (48) being generated by the system. BHA might be used in this regard as a fast H-atom donor to ensure the peroxy radicals formed are stabilized/trapped as the ROOH. Preliminary work in this author's laboratory suggests this approach may be possible. Further work is needed and other approaches could be effective.

Two final areas of investigation will be mentioned in closing. One last important area of research would be to better understand azonitrile-derived peroxy radical reactions in the limit of dilute azo compound and no substrates, other than water and acetonitrile and methanol cosolvents. This would lead to a "proof" of the mechanism by which methanol quenches the 2-cyano-2 propoxy radical activity (in the case of AIBN). Further work is also needed in terms of simple tests which help predict the potential of SET to dioxygen. Further investigations of possible correlations or "orthogonality" between CV data and azonitrile oxidation data may exist, and a unified approach to both data sets might provide a better insight into predicting SET to dioxygen.

These questions and topics will hopefully stimulate further thought and lead to additional work, approaches and insights into oxidative susceptibility testing.

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