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Inhibition of Soybean Lipoxygenase 1 by *N*-Alkylhydroxylamines†

Charles H. Clapp,* Amit Banerjee,‡ and Susan A. Rotenberg§
Department of Chemistry, Brown University, Providence, Rhode Island 02912
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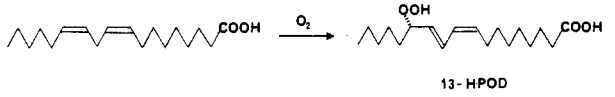
ABSTRACT: Micromolar concentrations of *N*-octylhydroxylamine dramatically increase the induction period in the conversion of linoleic acid to 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13-HPOD) catalyzed by soybean lipoxygenase 1. The induction period produced by *N*-octylhydroxylamine is abolished by 13-HPOD but not by the corresponding hydroxy acid. Addition of a catalytic amount of lipoxygenase to a mixture of 13-HPOD and *N*-octylhydroxylamine results in consumption of approximately 1 μmol of 13-HPOD/μmol of *N*-octylhydroxylamine present. These results can be explained by a model in which 13-HPOD oxidizes the enzyme from an inactive ferrous form to an active ferric form, as proposed by previous workers, and *N*-octylhydroxylamine reduces the enzyme back to the ferrous form. Consistent with this model, the ESR signal at $g = 6.1$ characteristic of ferric lipoxygenase is rapidly abolished by *N*-octylhydroxylamine and can be regenerated by 13-HPOD. These results provide additional support for earlier proposals that ferric lipoxygenase is the catalytically active form and also establish a novel method of inhibiting enzymes in this class. The octyl group of *N*-octylhydroxylamine appears to contribute to binding near the iron, since hydroxylamine and *N*-methylhydroxylamine do not extend the induction period. In the *n*-RNHOH series, activity passes through an optimum at R = decyl.

Soybean lipoxygenase 1 catalyzes the oxygenation of linoleic acid to 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13-HPOD)[1] [for reviews, see Axelrod (1974) and Veldink et al. (1977)].



13-HPOD

The enzyme will also oxygenate other fatty acids and fatty acid derivatives that have a *cis*,*cis*-1,4-diene unit that begins on the sixth carbon from the methyl terminus. Lipoxygenases are widespread in the plant kingdom, and lipoxygenase 1 is one of three isoenzymes that have been purified from soybeans. Interest in the mechanism and inhibition of lipoxygenases has been stimulated by recent discoveries of several physiologically and pharmaceutically interesting lipoxygenase reactions in mammalian arachidonic acid metabolism (Bailey & Chakrin, 1981).

Lipoxygenase 1 has a molecular weight of about 100 000 and contains one atom of non-heme iron per molecule (Chan, 1973; Roza & Francke, 1973; Pistorius & Axelrod, 1973). Removal of the iron results in loss of catalytic activity (Pistorius & Axelrod, 1974). The purified enzyme is ESR silent, and magnetic susceptibility measurements indicate that the iron is in the high-spin ferrous state (Slappendel et al., 1982b; Cheesbrough & Axelrod, 1983). Treatment of the enzyme with 1 equiv of its product, 13-HPOD, results in oxidation of the iron to a high-spin ferric form that exhibits an ESR signal near $g = 6$ and a weak ultraviolet absorption at 320 nm (De Groot et al., 1975a,b; Pistorius et al., 1976; Slappendel et al., 1981).

Under some assay conditions, lipoxygenase 1 exhibits a short induction period that appears to be due to activation of the enzyme by product, since no induction period is observed if low concentrations of 13-HPOD are present at the start of the reaction (Haining & Axelrod, 1958). The corresponding alcohol, 13-HOD, does not eliminate the induction period (Gibian & Galaway, 1976; Funk et al., 1981). The induction period can be extended by addition of glutathione peroxidase in the presence but not in the absence of glutathione, presumably due to consumption of 13-HPOD (Smith & Lands, 1972).

The apparent product activation can be reconciled with the physical studies by the hypothesis that the ferric enzyme is the catalytically active form (De Groot et al., 1975a; Pistorius et al., 1976). Further support for this notion comes from the observation that the spectroscopic features of the ferric enzyme can be eliminated by treatment with linoleic acid under anaerobic conditions (De Groot et al., 1975a; Egmond et al., 1977). Vliegenthart and co-workers have proposed a catalytic cycle consistent with these facts (De Groot et al., 1975a).

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* Address correspondence to this author at the Department of Chemistry, Haverford College, Haverford, PA 19041-1392.
‡ Present address: Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104.
§ Present address: Division of Molecular Biology and Virology, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.

[1] Abbreviations: 13-HPOD, 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid; 13-HOD, 13(*S*)-hydroxy-*cis*-9,*trans*-11-octadecadienoic acid; ESR, electron spin resonance; NMR, nuclear magnetic resonance; UV, ultraviolet; SDS, sodium dodecyl sulfate.

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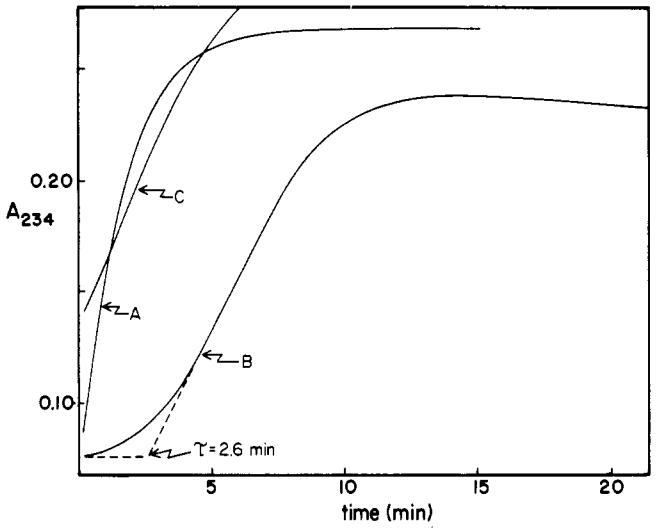


FIGURE 1: Effects of *N*-octylhydroxylamine on lipoxygenase turnover. (A) Linoleic acid (8.8 μM) plus lipoxygenase (2.6×10^{-3} μM) in 50 mM sodium borate buffer, pH 9.0. (B) Contents of (A) plus 5.0 μM *N*-octylhydroxylamine. (C) Contents of (B) plus 2.0 μM 13-HPOD. All reaction mixtures contained 0.6% v/v ethanol and were initiated by addition of enzyme.

In the course of investigating a variety of nonpolar compounds bearing functional groups that might interact with the iron in lipoxygenase, we discovered that *N*-octylhydroxylamine dramatically increases the induction period. In this paper we present evidence that this effect is due to reduction of the active ferric enzyme to the ferrous form. Our initial studies concerning the structural requirements for this novel mode of inhibition are also presented.

Materials and Methods

Lipoxygenase activity was assayed by monitoring the absorbance of 13-HPOD at 234 nm (ϵ_{max} = 25 000) in a Perkin-Elmer 552 UV–visible spectrophotometer. For determination of specific activities enzyme was incubated with 83 μM linoleic acid in 0.2 M sodium borate, pH 9.0, containing Tween 20 (23 mg/L) as described previously (Axelrod et al., 1981). One unit of activity is defined as the production of 1 μmol of 13-HPOD/min at 25 °C. Protein concentration was determined spectrophotometrically by using $\epsilon^{1\%}_{280nm}$ = 14.0 (Axelrod et al., 1981). Inhibition studies were carried out in 50 mM borate, pH 9.0, in the absence of Tween 20. Stock solutions of substrate and inhibitors were prepared in ethanol, and the final ethanol concentration in each reaction mixture was adjusted to 0.6% v/v.

Lipoxygenase 1 from soybeans obtained locally was purified by a published procedure (Axelrod et al., 1981) except that the first DEAE-Sephadex column was run with a less steep gradient and the second DEAE-Sephadex column was omitted. Preparations were >90% homogeneous by SDS–polyacrylamide gel electrophoresis, but the specific activities (100–125 units/mg) were considerably lower than the highest reported (180 units/mg) under the assay conditions we employ (Axelrod et al., 1981). Inhibition experiments carried out with type I and type V lipoxygenase obtained from Sigma Chemical Co. gave results in qualitative agreement with those obtained on more highly purified enzyme.

13-HPOD was prepared enzymatically from linoleic acid and reduced to 13-HOD with $NaBH_4$ (Gibian & Galaway, 1976). *N*-Methylhydroxylamine hydrochloride was obtained from Aldrich. Other *N*-alkylhydroxylamines were prepared by reduction of the corresponding oximes with sodium cyanoborohydride (Borch et al., 1971), recrystallized from hexane, and characterized by ^1H NMR and elemental analysis (C, H, and N). Melting points are listed in Table II.

ESR spectra were obtained on a Bruker ER-220D instrument interfaced with an ER-144 data system.

Table I: Induction Periods (τ) Observed at Different Concentrations of *N*-Octylhydroxylamine, Linoleic Acid, and Lipoxygenase[a]

linoleic acid (μM)	*N*-octylhydroxylamine (μM)	lipoxygenase (nM)	τ (min)
8.8		2.6	<0.2
8.8	3	2.6	1.4
8.8	5	2.6	2.6
8.8	8	2.6	5.0
8.8	8	5.2	3.7
17	8	2.6	1.5

[a] All reactions were carried out at 25 °C in 50 mM sodium borate buffer, pH 9.0, containing 0.6% v/v ethanol and were initiated by addition of enzyme.

Table II: Induction Periods (τ) Produced by *N*-Alkylhydroxylamines[a]

inhibitor	mp (°C)	concn (μM)	τ (min)
NH_2OH		10^3	<0.2
CH_3NHOH		10^2	<0.2
n-$C_8H_{17}NHOH$	74–75	5	3.1
n-$C_{10}H_{21}NHOH$	80.5–82	5	3.4
n-$C_{12}H_{25}NHOH$	87–88	5	2.4
$C_6H_5CH_2NHOH$	55.5–56.5	10^2	<0.2
n-$C_5H_{11}CH(NHOH)C_6H_5$	69–70	30	3.3

[a] Reactions were initiated by addition of lipoxygenase (0.014 unit) to solutions of linoleic acid (10 μM) and *N*-alkylhydroxylamine at the indicated concentration in 2.5 mL of 50 mM sodium borate, pH 9.0, at 25 °C.

Results

The effects of *N*-octylhydroxylamine on lipoxygenase turnover are illustrated in Figure 1. Curve A is the time course of formation of 13-HPOD (measured by its absorbance at 234 nm) when 2.6×10^{-3} μM lipoxygenase was incubated with 8.8 μM linoleic acid in 50 mM sodium borate buffer, pH 9.0. Under these conditions the induction period is too short to measure. Curve B was obtained from an identical reaction mixture that also contained 5 μM *N*-octylhydroxylamine, which produces an induction period of 2.6 min. The steady-state velocity is also reduced, and at the end of the reaction the absorbance at 234 nm slowly decreases. This last observation will be considered later. Curve C demonstrates that the presence of 2.0 μM 13-HPOD eliminates the induction period produced by *N*-octylhydroxylamine but does not restore the steady-state velocity to that in curve A. In contrast, the corresponding alcohol, 13-HOD, at 2.0 μM increases the induction period produced by 5 μM *N*-octylhydroxylamine to 3.0 min (data not shown) and causes a further decrease in the steady-state velocity.

The data in Table I demonstrate that the induction period is increased by increasing *N*-octylhydroxylamine concentration, decreasing enzyme concentration, and decreasing substrate concentration. The effects of varying the structure of the alkyl group are presented in Table II. Octanohydroxamate[2] is a competitive inhibitor of the enzyme ($K_i = 6 \times 10^{-5}$ M) as is the oxime of octanal ($K_i = 2 \times 10^{-4}$ M), but neither compound extends the induction period.

The data in each of Tables I and II were obtained on the same day by using the same set of enzyme, substrate, and buffer solutions. Under these conditions the magnitude of the

[2] A paper describing our work on the inhibition of lipoxygenase 1 by hydroxamic acids is in preparation.

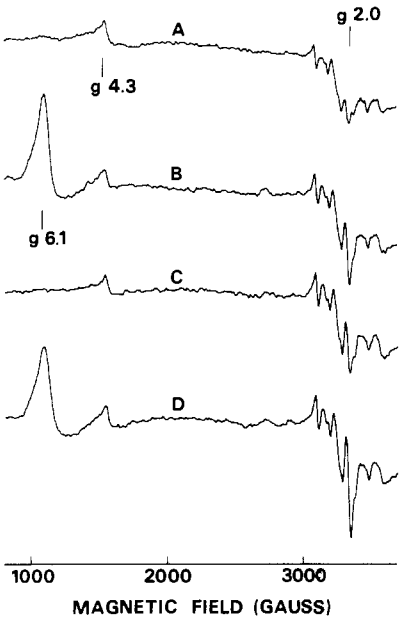
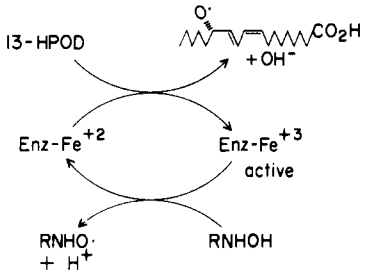


FIGURE 2: Effects of 13-HPOD and *N*-octylhydroxylamine on the ESR spectrum of lipoxygenase 1. (A) Lipoxygenase (0.114 μmol) in 0.2 mL of 50 mM sodium borate, pH 9.0. (B) Sample A plus 13-HPOD (0.050 μmol) and ethanol (2 μL). (C) Sample B plus *N*-octylhydroxylamine (0.060 μmol) and ethanol (2 μL). (D) Sample C plus 13-HPOD (0.070 μmol) and ethanol (2 μL). Each spectrum is the computed average of 10 scans obtained under the following conditions: spectrometer frequency 9.32 GHz; modulation frequency 100 KHz; modulation amplitude 2 G; microwave power 20 mW; temperature 120 K.

Scheme I



induction period is reproducible to ±0.25 min. However, considerable variability is observed from one set of experiments to another. For example, under the conditions of Figure 1 we have observed induction periods ranging from 2 to 5 min. Possible sources of this variability are considered under Discussion.

Since hydroxylamine derivatives are reducing agents (Smith, 1966), *N*-octylhydroxylamine could extend the induction period by reducing active ferric lipoxygenase to the ferrous form (Scheme I). This hypothesis was tested by the ESR experiments presented in Figure 2. In agreement with earlier reports (De Groot et al., 1975a,b; Pistorius et al., 1976), we find that treatment of native lipoxygenase with approximately 0.5 equiv of 13-HPOD results in the appearance of a signal at $g = 6.1$ (spectrum B) that is not present in the spectrum of the native enzyme (spectrum A).[3] As in earlier work,

[3] Previous workers have reported that treatment of lipoxygenase with more than one equivalent of 13-HPOD gives rise to additional ESR signals attributed to complexes of ferric lipoxygenase with 13-HPOD (DeGroot et al., 1975b). In order to avoid complications arising from the formation of these complexes, we used considerably less than 1 equiv of 13-HPOD to obtain spectrum B, particularly since the relatively low specific activity of our enzyme preparations creates an uncertainty as to the exact concentration of active enzyme. Subsequent experiments have shown that addition of approximately 0.75 equiv of 13-HPOD maximizes the signal at $g = 6.1$.

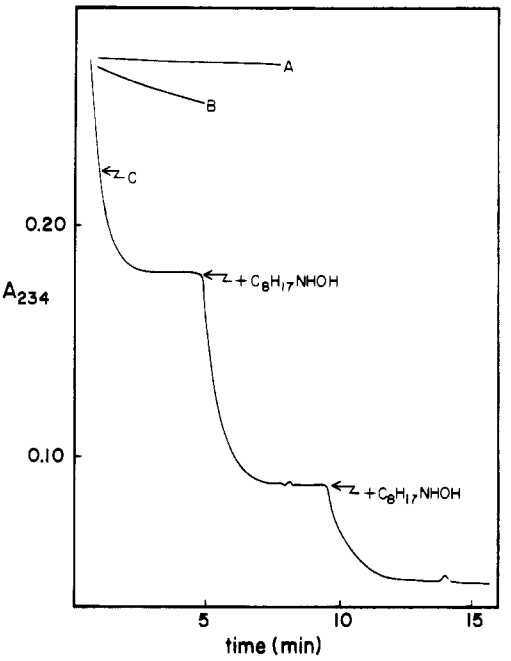


FIGURE 3: Time course of lipoxygenase-catalyzed reaction between 13-HPOD and *N*-octylhydroxylamine at 25 °C. (A) *N*-Octylhydroxylamine (4.0 μM) plus 13-HPOD (11 μM) in 50 mM sodium borate, pH 9.0. (B) Contents of (A) plus 2.6×10^{-3} μM lipoxygenase. (C) Contents of (A) plus 6.2×10^{-2} μM lipoxygenase. Aliquots of *N*-octylhydroxylamine, each corresponding to a final concentration of 4.0 μM, were added at the indicated points.

additional resonances are present: those near $g = 2$ are presumably due to contaminating metals such as Cu^{2+} and Mn^{2+} and that near $g = 4.3$ to a small amount of high-spin Fe^{3+} in a rhombic environment. Subsequent addition of *N*-octylhydroxylamine in 20% excess over the 13-HPOD added previously results in disappearance of the signal at $g = 6.1$ (spectrum C). The *N*-octylhydroxylamine was added at room temperature and mixed manually for about 30 s prior to freezing. If additional 13-HPOD is added (spectrum D), the $g = 6.1$ signal reappears.

Several aspects of the spectra in Figure 2 require comment. First, the spectra were obtained at 120 K, considerably above the range used by previous workers (15–77 K). We find that the appearance of spectrum B is unaltered by lowering the temperature to 8 K but that the signals broaden considerably above 135 K. Second, our spectra lack the signal at $g = 7.5$ that is present in most published spectra of ferric lipoxygenase and is believed to be due to a second form of the ferric enzyme (Slappendel et al., 1981). It has recently been reported that this signal is eliminated by low concentrations (0.05% v/v) of ethanol (Slappendel et al., 1982a); the presence of 1% ethanol (from the 13-HPOD stock solution) in our samples probably accounts for our failure to observe this signal. Finally, spectra B and D exhibit increased resonance near $g = 2$ relative to spectra A and C. It is not certain whether these changes are due to the protein or to the appearance of radicals in solution that had not proceeded to stable products prior to freezing.

Scheme I predicts that addition of lipoxygenase to a mixture of 13-HPOD and *N*-octylhydroxylamine should result in stoichiometric consumption of these two compounds. This would also account for the decrease in absorbance that occurs at the end of curve B in Figure 1. Figure 3 demonstrates that such a reaction occurs. Curve B was obtained by incubation of 2.6×10^{-3} μM lipoxygenase with 4.0 μM *N*-octylhydroxylamine and 11 μM 13-HPOD (concentrations similar

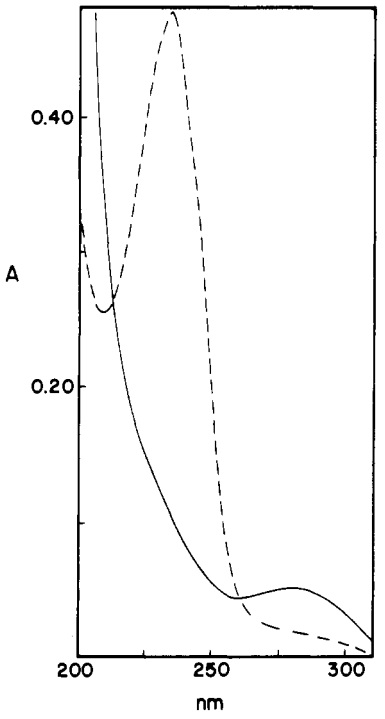


FIGURE 4: UV spectra of 18 μM 13-HPOD in 50 mM sodium borate, pH 9.0, before (---) and after (—) treatment with 25 μM *N*-octylhydroxylamine plus 1.3×10^{-2} μM lipoxygenase.

to those at the end of curve B in Figure 1), and curve C was obtained at 25 times higher enzyme concentration. In the absence of enzyme (curve A) or in the presence of heat-denatured enzyme the decrease in absorbance is barely detectable, and no decrease is observed in the absence of *N*-octylhydroxylamine. Curve C indicates that *N*-octylhydroxylamine is being consumed, since the decrease in absorbance stops but resumes upon addition of a second aliquot of *N*-octylhydroxylamine. A third aliquot of *N*-octylhydroxylamine produces a decrease in absorbance smaller than the first two, presumably due to complete consumption of 13-HPOD, since the reaction now does not resume upon further addition of *N*-octylhydroxylamine or enzyme. On the basis of the decreases in absorbance observed with the first two aliquots and the infinity absorbance at end of the reaction, it can be calculated that 0.89 μmol of *N*-octylhydroxylamine is consumed per micromole of 13-HPOD. The spectrum at the end of the reaction (Figure 4) shows complete loss of the diene chromophore of 13-HPOD, an increase in the end absorption, and a weak absorption near 280 nm. (The enzyme does not absorb significantly at its concentration in this experiment.) This spectrum will be considered further under Discussion.

Discussion

On the basis of the ESR results we conclude that the induction period produced by *N*-octylhydroxylamine is almost certainly due to the ability of this compound to reduce the essential iron in lipoxygenase from ferric to ferrous. Our ESR studies demonstrate that the proposed reduction can occur rapidly but do not rigorously establish that it occurs sufficiently rapidly to account for the observed induction period.

Jindal et al. (1970) have reported that reduction of ferricyanide by unprotonated hydroxylamine proceeds with a second-order rate constant of 0.63 M^{-1} s^{-1}, which indicates that this process is considerably slower than the reduction of lipoxygenase by *N*-octylhydroxylamine. As discussed below, the latter process is probably accelerated by binding of the octyl group to the enzyme and perhaps also by stabilization of nitrogen-centered radical intermediates by the alkyl substituent. In addition, reports (Slappendel et al., 1982a) that ferric lipoxygenase reverts to the ferrous form on storage suggest that the iron in this enzyme may be unusually susceptible to reduction.

As predicted by Scheme I, a reaction occurs when lipoxygenase is added to a mixture of 13-HPOD and *N*-octylhydroxylamine. We have not characterized this reaction in detail, but preliminary analysis of the product mixture indicates it to be complex. The ultraviolet spectrum obtained at the end of the reaction (Figure 4) exhibits a weak absorption near 280 nm which is likely due to 13-keto-9,11-octadecadienoic acid and 13-oxo-9,11-tridecadienoic acid formed by fragmentation of the alkoxide radical (Scheme I) produced by one-electron reduction of 13-HPOD (Verhagen et al., 1978; Garssen et al., 1971). However, on the basis of reported extinction coefficients (Garssen et al., 1971), the magnitude of the absorption at 280 nm indicates that only about 10% of the alkoxide radical decomposes by this pathway. Literature precedent (Hamberg, 1975; Dix & Marnett, 1983) suggests that the major fate of the alkoxide radical may be cyclization to an epoxyallylic radical which would give rise to products that do not absorb in the near-UV. Scheme I predicts that the consumption of 13-HPOD and *N*-octylhydroxylamine should exhibit a 1:1 stoichiometry. The experiment depicted in Figure 3, in which the concentration of 13-HPOD was determined spectrophotometrically, as well as other experiments, in which the concentration of 13-HPOD was determined iodometrically (Gibian & Galaway, 1976), indicates that slightly less than 1 μmol of *N*-octylhydroxylamine is consumed per micromole of 13-HPOD. This deviation from 1:1 stoichiometry may be due to secondary reactions between the starting materials and the radicals produced by the reactions in Scheme I.

The lipoxygenase-catalyzed reaction between *N*-octylhydroxylamine and 13-HPOD accounts for the fact that curve B in Figure 1 reaches a maximum below that of curve A and then decreases. The maximum absorbance of curve B is 84% that of curve A, which is consistent with consumption of 1.4 μM 13-HPOD and a slightly smaller amount of *N*-octylhydroxylamine. This means that about 3.7 μM *N*-octylhydroxylamine is still present when the maximum is reached, and the enzymatic reaction between this material and 13-HPOD causes the absorbance to fall. The inhibition of the steady-state rate could be due either to binding of *N*-octylhydroxylamine at the active site or to the fact that some of the enzyme is in the reduced form. Steady state is apparently achieved when the concentration of 13-HPOD reaches about 2 μM; increasing the enzyme concentration or substrate concentration below saturation would be expected to hasten the attainment of this product concentration and therefore reduce the induction period. Over the limited concentration range that we have investigated (Table I) these expectations are borne out. Previous workers (Smith & Lands, 1972) have found that the induction period observed in the absence of *N*-octylhydroxylamine is increased by increasing the substrate concentration, but these experiments were carried out at high substrate concentration (>100 μM) where the kinetics are complicated by substrate inhibition (Egmond et al., 1976). As expected, 13-HPOD but not 13-HOD eliminates the induction period. The small increase in the induction period produced by 13-HOD is also observed in the absence of *N*-octylhydroxylamine (Gibian & Galaway, 1976) and may be due to binding of 13-HOD to the ferrous enzyme in competition with 13-HPOD.

The octyl group of *N*-octylhydroxylamine appears to contribute to binding of this compound near the iron since neither hydroxylamine nor *N*-methylhydroxylamine produces induction periods. In the *n*-alkyl series there is an apparent optimum activity at 8–10 carbons (See Table II. In a separate experiment it was shown that *N*-hexylhydroxylamine is about half as active as the *N*-octyl derivative.) This optimum could be due to an interaction between the alkyl group of the inhibitor and the binding site for the alkyl terminus of substrates, but an optimum at slightly shorter chain length would have been expected. We have not ruled out the possibility that the apparent optimum is due to aggregation of the longer chain derivatives resulting in lowering of their effective concentrations.

Since lipoxygenase apparently requires activation by its product, an important question arises as to how the reaction gets started. One obvious possibility is that enzyme preparations contain a small amount of the ferric enzyme, which produces 13-HPOD that activates the remaining enzyme. Alternatively, the reaction could be initiated by low levels of contaminating 13-HPOD in the substrate or the presence of adventitious oxidants in the assay mixture capable of slowly oxidizing the enzyme. Variability in the levels of ferric enzyme, contaminating 13-HPOD, or adventitious oxidants is likely responsible for the observed variability in the length of the induction period, and attempts to distinguish among these possibilities are in progress. Egmond et al. (1977) have presented some evidence for an additional mode of activation involving a weak catalytic activity of the ferrous enzyme. Experiments at high enzyme concentration and high concentrations of *N*-octylhydroxylamine may be helpful in evaluating the activity of the ferrous enzyme.

The work presented here provides additional support for earlier proposals that ferric lipoxygenase is the fully active form. Furthermore, investigating the effects of *N*-alkylhydroxylamines on the turnover of mammalian lipoxygenases may provide a simple test for whether these enzymes require activation to the ferric state. Finally, the work demonstrates a new method for inhibiting lipoxygenases. Since the utility of hydroxylamine derivatives for in vivo applications is likely to be limited by their reactivity and toxicity, we are searching for less reactive compounds that inhibit soybean lipoxygenase by the same mechanism.

Registry No. 13-HPOD, 33964-75-9; *n*-$C_8H_{17}NHOH$, 2912-95-0; NH_2OH, 7803-49-8; CH_3NHOH, 4229-44-1; *n*-$C_{10}H_{21}NHOH$, 26228-72-8; *n*-$C_{12}H_{25}NHOH$, 95216-86-7; $C_6H_5CH_2NHOH$, 622-30-0; *n*-$C_5H_{11}CH(NHOH)C_6H_5$, 95216-87-8; Fe, 7439-89-6; lipoxygenase, 9029-60-1.

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