Reports

Mechanisms of Nitrogen Dioxide Reactions: Initiation of Lipid Peroxidation and the Production of Nitrous Acid

Abstract. *The reactions of nitrogen dioxide with cyclohexene have been studied as a model for the reactions that occur between nitrogen dioxide in smoggy air and unsaturated fatty acids in pulmonary lipids. As predicted from earlier studies at high nitrogen dioxide concentrations, this gas reacts with cyclohexene predominantly by addition to the double bond at nitrogen dioxide concentrations of 1 percent (10,000 parts per million) to 40 percent in nitrogen; in the presence of air or oxygen, this reaction initiates the autoxidation of the alkene. However, at concentrations below 100 parts per million in nitrogen, nitrogen dioxide reacts with cyclohexene almost exclusively by abstraction of allylic hydrogen; this unexpected reaction also initiates the autoxidation of the alkene in the presence of oxygen or air, but it leads to the production of nitrous acid rather than of a product containing a nitro group attached to a carbon atom. The nitrous acid can react with amines to produce nitrosamines. Moreover, the nitrite ion produced by the hydrogen abstraction mechanism would be expected to diffuse throughout the body, unlike nitrated lipids that would be confined to the pulmonary cavity. These findings have been confirmed with methyl oleate, linoleate, and linolenate; some of the kinetic features of the nitrogen dioxide–initiated autoxidation of these unsaturated fatty acids have been studied.*

Nitrogen dioxide, produced as an environmental contaminant when organic compounds undergo combustion (*1, 2*), is one of the most damaging pollutants. Nitrogen dioxide ($\cdot NO_2$) is a free radical and reacts with both alkanes and alkenes at 25°C by free-radical mechanisms (*3–8*). Both in vitro and in vivo studies have shown that NO_2 initiates the autoxidation of unsaturated fatty acids in lipids (*9*), and this lipid peroxidation process causes the destruction of pulmonary lipids (*9, 10*) and leads to membrane damage and cell death (*11*).

Organic chemists have demonstrated that NO_2 adds to alkenes (*3–5, 8, 12*); thus it is usually assumed that NO_2 initiates lipid autoxidation by addition to the double bonds of the unsaturated fatty acids, as shown in Eq. 1:

$$NO_2 + -\underset{|}{\overset{|}{C}}=\underset{|}{\overset{|}{C}}- \underset{k_2}{\overset{k_1}{\rightleftharpoons}} O_2N-\underset{|}{\overset{|}{C}}-\underset{|}{\overset{|}{C}}\cdot \qquad (1)$$

I

The organic studies were conducted at low temperatures and at high concentrations of NO_2 (*3, 4*), and relatively little is known about the reaction mechanism of NO_2 with alkenes at ambient temperature and below 1000 parts per million (ppm). For this reason, we have reexamined the mechanism of the initiation of autoxidation of alkenes by NO_2 (*13*). We carried out detailed studies in which cyclohexene was used as the substrate, since, with this symmetrical *cis* alkene, all the products from the NO_2 addition and NO_2-initiated autoxidation reactions can be identified. Less detailed work was done on a series of alkenes and polyenes, including unsaturated fatty acid esters.

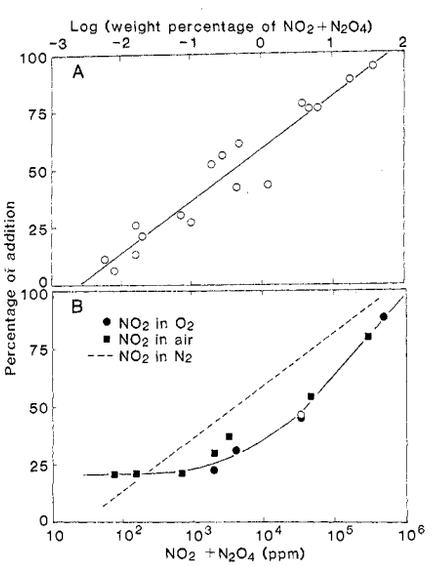

Fig. 1. The mole percentage of addition, calculated (*13*) as the ratio of the sum of addition products to the sum of all products formed by NO_2 initiation at 30°C, is plotted as a function of the concentration of nitrogen oxides in the carrier gas, either (A) N_2 or (B) air or O_2. [The carrier gas is blown past a reservoir containing N_2O_4. At low concentrations, N_2O_4 dissociates completely to NO_2; at higher concentrations both species are present (*14*).]

In our studies we used 6.6M solutions of cyclohexene in hexane; evidence in the literature indicates that only free-radical reactions occur in such solutions (*4–6*). A carrier gas (N_2, air, or O_2) containing NO_2 [and its dimer, dinitrogen tetroxide (N_2O_4) (*14*)], was bubbled through the solutions at 30°C. (This two-phase system was meant to provide a simplified model for pulmonary exposure conditions.) The resulting products were identified by a comparison of their characteristic spectra (nuclear magnetic resonance and infrared) or their gas-chromatographic retention times, or both, to those of known compounds (*13*).

Our data demonstrate that the principal initiation mechanism of NO_2 changes from addition to a double bond to abstraction of an allylic hydrogen (Eq. 2) as the NO_2 concentrations reach the low parts-per-million range:

$$NO_2 + -HC=CH-CH_2- \rightarrow HONO + -\dot{C}H-\dot{C}H-\dot{C}H- \qquad (2)$$

II

The two initiation mechanisms are distinguishable because each forms a unique set of products. Radical **I** is produced in Eq. 1; this species can react with another NO_2 to form a dinitro or a nitro-nitrite compound, or with O_2 (if air is present) to give 2-nitro-nitrates, 2-nitro-hydroperoxides, and other oxygenated compounds. Nitrous acid (HONO) and allylic radical **II** are produced in Eq. 2; radical **II** can combine with NO_2 to give an unsaturated nitro or nitrite compound or with O_2 to give the allylic hydroperoxide or nitrate ester, as well as products of autoxidation.

The data plotted in Fig. 1 illustrate the dependence of the initiation mechanism on the NO_2 concentration. In the absence of O_2 (Fig. 1A), a linear relationship exists between the percentage of addition and the logarithm of the nitrogen oxide concentration over the extremely broad concentration range studied. In the presence of O_2 (Fig. 1B), the percentage of addition also decreases as the NO_2 concentration is reduced; the curvature in Fig. 1B is discussed below.

We suggest the following explanation for the decreasing amounts of addition

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and increasing amounts of hydrogen abstraction that occur as the NO_2 concentration is decreased. Addition, the thermodynamically favored process (*15*), is reversible (Eq. 1) (*16, 17*). In contrast, hydrogen abstraction is not; the HONO formed in Eq. 2 either is swept out of the reaction in the carrier gas or decomposes to nitrogen oxides and water (Eq. 3) (*18*), thus preventing the reversal of Eq. 2:

$$2\ HONO \rightarrow NO + NO_2 + H_2O \quad (3)$$

Therefore, at high concentrations of a radical-trapping species (NO_2, N_2O_4, or O_2) intermediate **I** gives addition products, but at low concentrations of trapping species addition reverses and hydrogen abstraction is the net process that is observed. Equation 4 illustrates this competition, with O_2 as the radical-trapping species:

$$NO_2 + -\underset{|}{C}=\underset{|}{C}- \underset{k_2}{\overset{k_1}{\rightleftharpoons}} O_2N-\underset{|}{C}-\underset{|}{C}\cdot \quad \mathbf{I}$$

$$\xrightarrow[O_2]{k_3} O_2N-\underset{|}{C}-\underset{|}{C}-OO\cdot \quad \mathbf{III} \quad (4)$$

At low concentrations of the trapping species, radical **I** is trapped more slowly by O_2 and a larger fraction of **I** reverts to reform the alkene; therefore, less addition occurs and hydrogen abstraction becomes favored (*19, 20*).

At low NO_2 concentrations (Fig. 1B) a slightly larger amount of addition occurs when O_2 is present. Thus, as expected, the mixture of NO_2 and O_2 traps radical **I** better than NO_2 alone when NO_2 concentrations are low.

When O_2 is present, some of the products result from the NO_2-initiated autoxidation of cyclohexene (*13*). At low concentrations of NO_2, the kinetic chain length is long and 90 percent of the products result from the chain autoxidation process. The data in this low NO_2 range can be corrected for the products due to this chain reaction (*13*), and these corrected data (Fig. 1B) show that more addition occurs in the presence of O_2; however, the change is small. Analysis shows that the reversal of **I** is fast and that the trapping of **I** by O_2 (Eq. 4) is not quantitative [because of the large values for k_2 and the low concentration of O_2 in the liquid phase (*13*)]. These data suggest that at low concentrations of NO_2, pulmonary concentrations of O_2 may not prevent the reversal of **I**; thus, hyrdogen abstraction probably predominates in reactions of NO_2 with unsaturated fatty acids in vivo.

At high concentrations of NO_2, this gas is the predominant radical-trapping species in solution (*21*), and we would expect similar addition-abstraction ratios in N_2, air, or O_2. However, at high concentrations of NO_2 some 10 to 20 percent more addition occurs in N_2 than in O_2 or air (Fig. 1B). We believe that this is an artifact due to the production of abstraction products by autoxidation. (The hydrogen abstraction products are identical, regardless of whether NO_2 or an autoxidation chain species does the abstracting.) Above 1 percent (10,000 ppm) NO_2 in air or O_2, the kinetic chain length of the autoxidation is approximately one (*13*); therefore, the total yield of products does not indicate the extent to which an autoxidation chain contributes to product formation, and the products cannot be corrected for those due to autoxidation as was done above.

Table 1. Percentage of hydrogen abstraction occurring during NO_2-alkene reactions. Reactions were done in a sealed vessel at 30°C with 0.5 to 1 percent (weight-volume) NO_2 in N_2. The percentage of hydrogen abstraction is inferred from the yield of water (Eq. 3). This is an indirect and approximate method, but it gives data for cyclohexene in satisfactory agreement with the more exact product-analysis method discussed above. For discussion, see (*13*).

Alkene	Hydrogen abstraction (%)
Methyl linolenate (18:3)	71
Methyl linoleate (18:2)	67
Cyclohexene	56*
1,5,9-Cyclododecatriene	52
1-Hexadecene	38
Methyl oleate (18:1)	36

*Value agrees with the value obtained by product study (Fig. 1A).

We have studied NO_2-initiated autoxidation of methyl oleate (18:1, 18 carbons and one double bond), methyl linoleate (18:2), and methyl linolenate (18:3), using conditions similar to those in the cyclohexene study. Using classical inhibitor methods (*22*), we find that each absorbed NO_2 initiates one kinetic chain (*13*), indicating that NO_2 behaves as a simple, free-radical initiator. At the NO_2 concentrations used in the kinetic studies, 0 to 70 ppm, the cyclohexene data discussed above predict hydrogen abstraction to be the predominant initiation mechanism. In support of this, we find that both methyl linoleate and linolenate react predominantly by hydrogen abstraction (Table 1) at concentrations 0.5 to 1 percent NO_2 in N_2. Hydrogen abstraction predominates for these polyunsaturates even more than for cyclohexene since they have more reactive, doubly allylic hydrogens. Perhaps because of their acyclic structure and a resulting increase in steric hindrance about their allylic hydrogens, methyl oleate and 1-hexadecene react relatively less by the hydrogen abstraction mechanism than cyclohexene and 1,5,9-cyclododecatriene (Table 1).

At low concentrations of NO_2, all three unsaturated fatty acid esters, like cyclohexene, shift their reaction mechanisms more in favor of hydrogen abstraction. Since addition incorporates nitrogen into the fatty acid but hydrogen abstraction does not (Eqs. 1 and 2), we have determined the percentage of addition at low part-per-million levels by analyzing the fatty acids after reaction for nitrogen incorporation. These elemental analysis data indicate that below 1000 ppm NO_2 all the fatty acid esters, including even 18:1, react predominantly by hydrogen abstraction (*13*).

Both addition and hydrogen abstraction by NO_2 result in the initiation of autoxidation of the alkene or lipid. However, an important consequence of hydrogen abstraction in biological systems is the production of HONO in the lipid-rich regions of cell membranes. It had previously been thought that nitrosation of amines by HONO required protic media (*23*), but Mirvish *et al.* have shown that nitrosation can occur in aprotic media as well (*24*). Nitrogen oxides have also been shown to nitrosate amines both in vivo and in vitro in protic media (*25*).

We find that nitrosation occurs under the aprotic conditions of our autoxidation reactions. Dicyclohexylamine ($10^{-3}M$) was added to neat 18:1 and 18:2 fatty acid esters and exposed to 60 ppm NO_2 in air; a solution of $10^{-3}M$ amine in hexadecane was used as a control experiment. Analysis for nitrosamine (*26*) shows that the rate of nitrosation of amine is nearly doubled in the fatty acid ester solutions, an indication that HONO formed by the NO_2–unsaturated ester reactions is participating in the nitrosation of the amine. Thus, formation of HONO by NO_2-alkene reactions in the lipid regions of lung tissue and in the unsaturated oils of food could lead to nitrosation of amines.

The mechanism that we have proposed for NO_2-lipid reactions suggests a rationalization for the surprising data of Goldstein *et al.* (*27*). These workers used radiolabeling to show that NO_2 inhaled by primates is not localized in the lung but is distributed throughout the entire body (*27*). To explain this they suggest that the reaction of NO_2 with pulmonary water might produce nitrite and nitrate ions that enter the bloodstream. Howev-

er, two alternative explanations exist. One, which appears unlikely from the recent work of Parks *et al.* (*28*), is that the unchanged nitrogen oxides enter the bloodstream and react with hemoglobin to form a nitrogen oxide-iron complex (*29*). Alternatively, our data (*30*) as well as the *in vivo* data of Thomas *et al.* (*9*) show that NO_2 initiates lipid peroxidation even in aqueous systems. Addition of NO_2 would produce a lipid-bound nitro group; however, the hydrogen abstraction mechanism suggested here converts NO_2 to nitrite ions that would be carried throughout the body, as shown by Goldstein *et al.* (*27*).

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Mechanism of Single-Layer Graphite Oxidation: Evaluation by Electron Microscopy

Abstract. *Etch-decoration reveals that the rate of removal of carbon atoms exposed at monolayer steps on graphite surfaces depends on the population density of these edge atoms (the rate is higher at a low-density surface) and that carbon removal continues for a prolonged period after the oxygen supply in the gas phase has been shut off. The edge carbons are removed by both oxygen from the gas phase and oxygen in the adsorbed oxides which migrate from the neighboring basal carbon atoms.*

The first step in all gas-solid and solid-catalyzed reactions is the adsorption of the gaseous molecules on the solid surface. It is known that only on specific active surface sites can the adsorbed molecules react to form products, which may then desorb to the gas phase. This general class of reaction mechanism is known as the Langmuir-Hinshelwood (LH) mechanism, which has been a cornerstone model in catalysis and heterogeneous kinetics. The Michaelis-Menten mechanism for enzymatic reactions is synonymous with this mechanism. More recently, information on the mobility of the adsorbed species on the surface has been accumulating. A fundamental question, consequently, is how the mobile adsorbed species participates in the reaction. This question has been challenging enough that numerous indirect evidence for such a mechanism has been published (*1*). Along this line, we have measured the rate of removal of carbon atoms by oxygen on the monolayer steps on graphite, which are the active sites. We have found that the rate of removal, in atoms per active site per second, depends to a large extent on the population density of the active sites; the rate declines as the population density of these sites increases and carbon removal continues for a prolonged period after the O_2 supply in the gas phase has been cut off. The number of nonactive carbon sites was much greater than the number of active sites (by about 10^5 to 10^8). Our results show that the carbon at an active site is removed by two mechanisms: (i) direct reaction with O_2 in the gas phase or the LH mechanism and (ii) reaction with the migrating oxygen atoms of the surface oxides which are first dissociatively chemisorbed on the nonactive sites.

The experimental technique consists of etch-decoration followed by examination with transmission electron microscopy (TEM). The technique, described elsewhere (*2*), consists of cleaving single crystals (natural graphite from Ticonderoga, New York) to a thickness of a few hundred angstroms, etching the graphite in a gas (in our case, 20 percent O_2 in argon at 1 atm) which expands the surface vacancy to create a pit one atomic layer deep, decorating the edge of the pit with gold nuclei, and examining with TEM. The radius of the pit is proportional to the time of etching. The atoms on the edge of the pit are the active sites. From the pit growth rate, we are able to calculate the rate of removal of carbon atoms per active site.

The reaction with O_2 starts from the residual vacancies present on the basal surface. The density of the natural vacancies can be counted as the ring densi-

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