

Absorption and metabolic fate of ozone – the molecular basis of ozone-induced toxicity

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It is the purpose of this section to define and analyze the currently available data concerning the chemical basis of the reactivity of ozone with biological molecules. The material also illustrates some of the consequences of ozone reactivity for the maintenance of biochemical processes and cell viability and phenotype. Ultimately, the goal is a better appreciation of both the complexity of the molecular mechanism(s) through which ozone executes its pulmonary toxicity and how much remains to be investigated. For this purpose the review has been divided into several parts (figure 1).

The first part deals with the close interplay between ozone, a nonradical molecule, and reactive oxygen metabolites, such as superoxide ($\cdot\text{O}_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot\text{H}$), as well as other free radicals (figure 1). This description is essential, as many of the consequences of the exposure of biological material to ozone can be wholly or partially due to the conversion of ozone into other reactive species upon aqueous dissolution. The first section also details the limited data available on the absorption of ozone into the lung.

The second part deals with the chemical reactivity of ozone and progeny reactive species. It also shows how such species form the basis for the reaction of ozone with biological molecules, particularly with lipids, proteins, and nucleic acids.

The third part discusses the potential of such alterations to important biological molecules interfering with biochemical processes in cells, and therefore leading to altered cell phenotype, cytotoxicity, and genotoxicity.

It is not the purpose of this review to provide a comprehensive description of all of the literature on the subject. However, selected examples have been used to illustrate each point raised. At the end of each part, the current level of understanding is briefly summarized and specific areas of research are suggested which may, in the future, provide useful information, particularly with regard to possible sensitive biomarkers of exposure to ozone in humans.

The purpose of this structured approach is to put the complex chemical and biochemical events occurring in response to ozone at the molecular and cellular levels into perspective to help the reader evaluate data on the effects of ozone on more complex biological systems, such as the lung. An understanding of the initial biochemical events through which ozone exposure causes

alterations to cellular function is a prerequisite for (i) an understanding of how ozone affects normal lung physiology, (ii) the interpretation of biochemical effects resulting from prolonged ozone exposures, (iii) the identification of risk factors in humans, and (iv) the development of biomarker technology to detect biological responses and early signs of detrimental health effects.

Chemical relationships between ozone and reactive oxygen metabolites

Many early studies have clearly indicated that ozone toxicity can be explained in terms of free radical reactions. Indeed, ozone was considered to have radiomimetic properties and therefore to induce the generation of reactive species and free radicals and cause the induction of chromosome aberrations and facilitate interactions with antioxidant defense systems (1–6). Nevertheless, ozone is not a free radical, as is molecular oxygen. But it does have some of the properties of such a

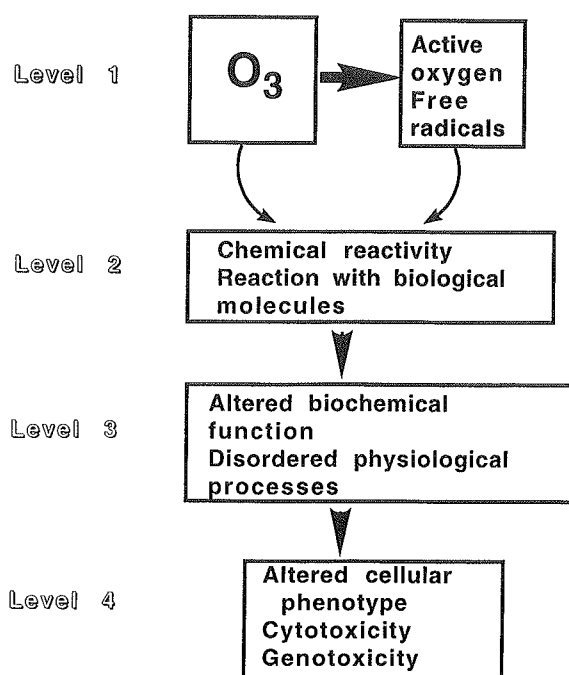


Figure 1. Schematic representation of the molecular basis of ozone toxicity.

radical, and it is clearly reactive, with a redox potential of +2.07 V (7).

The work of Taube (8) and Baxendale & Wilson (9) led Hoigne & Bader (10) to suggest that many of the actions of ozone in solutions of physiologically relevant pH are due to its decomposition to the hydroxyl radical (10). Several processes are thought to be involved (figure 2) in which the decomposition is initiated by hydroxyl ions (11), the presence of hydrogen peroxide (12), or irradiation with ultraviolet light (10, 13).

A consensus of opinion now exists that the reaction of ozone with organic molecules in aqueous solutions of near neutral or alkaline pH is a combined effect of the reactivity of ozone itself and the reactivity of a number of other reactive oxygen metabolites, particularly that of the hydroxyl radical. The hydroxyl radical is much more reactive than the parent ozone molecule, and it is more indiscriminate in its reactions. This relationship provides one of the biggest barriers to understanding the molecular basis of ozone toxicity, but for the purposes of this review, when the reactivity of ozone is considered in biological systems, the involvement of other oxidants is implicit.

Absorption of ozone into the lung

Before considering the biological effects of ozone in the lung, one must first address the question of the extent to which ozone is absorbed from the atmosphere into the lung.

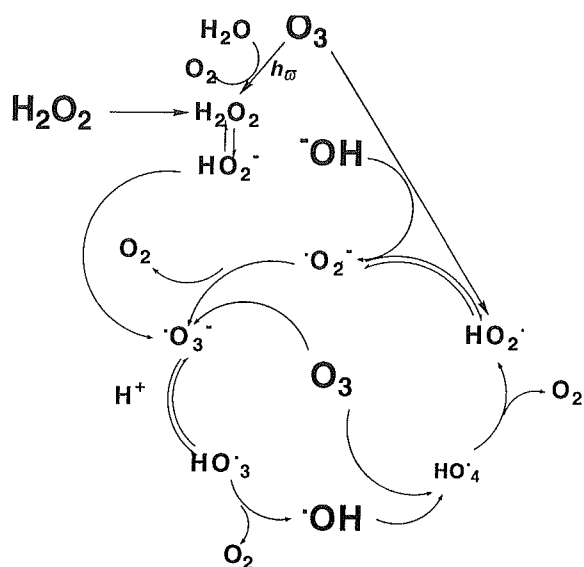


Figure 2. Cyclic decomposition of ozone catalyzed by the hydroxyl ion, hydrogen peroxide, or ultraviolet irradiation.

Elegant experiments by Ultman et al (14), using morphometric analysis of inhaled and exhaled ozone, have shown that the penetration of the gas into the lungs is mainly dependent on the rate of airflow, the tidal volume, and the manner of breathing. Thus during quiet, mainly nasal breathing most ozone is absorbed extrathoracically. On the other hand, during exercise, proportionately more ozone is absorbed by the lower respiratory airways. These authors have also shown that the rate of ozone diffusion around the airways of the lung and eventual chemical or absorption processes are proportionate to the concentration of ozone in the inhaled air (14). These morphometric studies with humans indicate that a large portion of inhaled ozone, as much as 90%, is absorbed by the total respiratory tract, with a large proportion, 30 to 40%, being absorbed by the nasal or oral airways. In addition, these studies indicate that the centriacinar region of the bronchial tree receives the largest dose of ozone when the gas is inhaled. However, these studies do not reveal the metabolic fate of the gas in the lung.

The close interplay between ozone and reactive oxygen metabolites, as well as its reactivity with biological molecules, presents a quandary with respect to the degree of penetration of inhaled ozone into and across the pulmonary epithelium, into the interstitium, and then out into systemic circulation. Limited experimental evidence is available to suggest that these considerations are of primary importance if the mechanisms by which ozone elicits its pulmonary toxicity are to be understood fully. Pryor has postulated that ozone is absorbed into the body via the lungs, but he suggests that ozone itself does not penetrate the epithelial lining fluids in the airways, except in areas of minimal lining thickness or lack of surfactant. Thus it was proposed that ozone-induced cytotoxicity and alterations to lung function result from the reactions of more stable, but nonetheless reactive products of the interaction of ozone with biomolecules, such as lipids. These species, among others, would then propagate the ozone signal into the underlying cellular layers of the lung (figure 3).

In addition, there has been limited use of isotopically-labeled ozone, $^{18}\text{O}_3$, in clinical experiments. Recently it was shown that the isotope was recoverable in lavage cells after inhalation and that the recovery was increased by exercise (17). It is clear that this approach presents definite possibilities to identify the extent of the penetration of ozone into the tissues and to identify the eventual reaction products of its interaction with biological macromolecules, particularly those of lipid origin.

In summary, present knowledge suggests that although most inhaled ozone is absorbed into the respiratory tract during quiet breathing, the molecule is itself not appreciably absorbed into the body via the lungs.

Reactivity of ozone with biological molecules

General reactivity with organic molecules

Ozone reacts readily with unsaturated compounds such as alkenes, alkynes, and aromatic systems (18), as well as with aromatic and aliphatic amines (19). In general, the aromatic systems should be activated towards electrophilic substitution. Such compounds include phenols and aromatic ethers (20). Despite the importance of studying the reactions of ozone with aromatic compounds, particularly from the point of view of ozonolysis processes in water purification, few studies have investigated the by-products of such reactions. However, it appears that, irrespective of the aromatic systems studied, ring-hydroxylations and ring-opening reactions are prevalent (21), in a manner reminiscent of the direct reaction of aromatic compounds with hydroxyl radicals.

Reaction with biological antioxidants

In the context of reactivity it is natural initially to define how ozone interacts with endogenous electron donors which function as antioxidants. One of the best studied targets in this context is glutathione. This thiol-containing tripeptide, along with the associated activities of enzymes such as the glutathione peroxidases, glutathione transferases, and glutathione reductase, represents one of the major biological antioxidant defense mechanisms, which is directed primarily against reactive oxygen metabolites, particularly against oxygen peroxide. The system is also directed against the accumulation of secondary products of the interaction of reactive oxygen metabolites with biological macromolecules, such as the lipid peroxides and the reactive aldehydes produced during lipid peroxidation (22).

Ozonation of simple glutathione-containing solutions causes rapid oxidation of the molecule (23). On the other hand, and, surprisingly, there have been no reports of an observation of acute oxidation of cellular glutathione upon the exposure of isolated cells to ozone. This lack may be due to the rapid reduction of oxidized glutathione by glutathione reductase at the expense of cellular NADPH (nicotinamide adenine dinucleotide phosphate), an event which might only be overwhelmed by exposures to extremely high and irrelevant concentrations of ozone. However, limited work with isolated A-549 epithelial cells from the lung has clearly indicated that cellular glutathione is a front-line antioxidant against ozone, as the depletion of the tripeptide prior to exposure to ozone enhances the acute cytotoxicity of the gas (24). This work also strongly suggested that the protective function of glutathione in these cells is primarily due to its serving as a co-factor for the glutathione transferase-dependent detoxication of lipid ozonides, rather than in

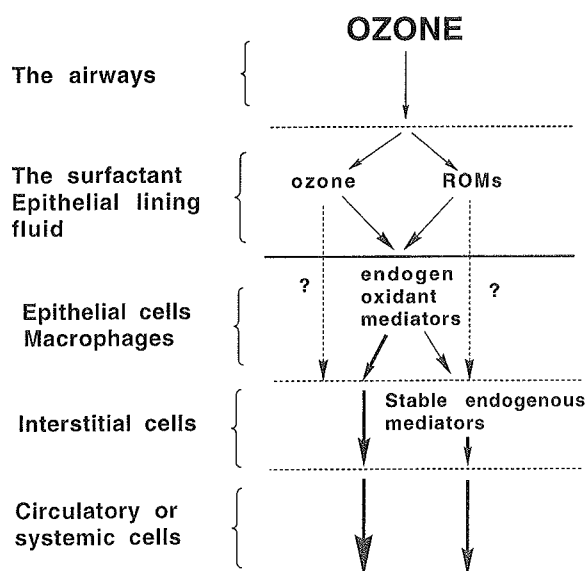


Figure 3. Potential amplificatory cascade of reactive and stable endogenous mediators derived from ozone in the lung. (ROM = reactive oxygen metabolites)

the glutathione peroxidase-dependent detoxication of hydrogen peroxide or lipid peroxides (25).

In contrast to these effects of acute exposure *in vitro*, chronic exposure of animals to ozone has been shown to induce the levels or activities of various components of the pulmonary glutathione-dependent antioxidant defense. Increases in pulmonary glutathione (26–29), glutathione peroxidase (27, 28, 30–32), and glutathione reductase (31) activities have been shown, and it has been suggested that this is an adaptive response aimed at increasing the antioxidant protection of the lung during prolonged exposure. On the basis of such observations, it was envisaged that fluxes in the glutathione system could provide a marker of pulmonary exposure to ozone. This possibility is, however, complicated by certain factors. First, in addition to the obvious dose-response relationships, the extent of these inductions is often time- and species-dependent. For instance, Tepper et al (29) noted that the increase in lung glutathione which occurred in response to ozone was maximal during the first 2 d of exposure and thereafter declined to control levels by day 4. Similarly, Ichinose and his co-workers clearly showed that nonprotein thiol (>95% glutathione) and glutathione peroxidase activity increases in rat lung in response to ozone over a two-week period, while, in response to similar levels of ozone, the corresponding parameters in guinea pig were shown to decrease (27). This difference suggests interspecies variation for the response. A further complication arises from the fact that the inductions are often quantitatively minor and their significance may decrease or disappear if the data are corrected for the cell proliferation and increased DNA (deoxyribonucleic acid) syn-

theses which occur in the lung in response to ozone (33). Thus the induction of glutathione peroxidase and reductase noted in rat lungs in response to exposure to ozone for 3 d was not significantly different from that of controls when corrected for increases in cell number in the lung (31).

In addition to glutathione, ascorbate is another major water-soluble component of the intracellular antioxidant defense network (22). Pryor (16) has shown that ozone reacts efficiently with ascorbate in simple solutions. Nevertheless, controlled studies on the effects of the acute exposure of cells to ozone *in vitro* on ascorbate levels are absent from the literature. In parallel with glutathione, pulmonary ascorbate levels have been shown, however, to be enhanced by the chronic exposure of animals to ozone (26, 29).

Finally, the major protective antioxidant in biological membranes is vitamin E. Although vitamin E reacts directly with ozone, the reaction rate is inferior to that between ozone and ascorbate (16). Few controlled studies have been performed on the effect of ozone exposure on vitamin E levels in cells *in vitro* or in animals *in vivo*. Elsayed and his co-workers (34) have shown that vitamin E levels increase in the lung of vitamin E-supplemented rats in response to ozone exposure over several days. The importance of vitamin E in the protection of the lung from the effects of ozone is more clearly demonstrated by the observation that the depletion of vitamin E from rats prior to the exposure to ozone clearly exacerbates the toxicity of the gas (33, 34). The importance of vitamin E at the cellular level will become clear as the effects of ozone on lipid metabolism in cells are discussed.

In addition to being present within the intracellular milieu, both glutathione and ascorbate occur at relatively high concentrations in the epithelial lining fluid of the lung. Thus Pryor (25) has suggested that, due to the relatively poor penetrability of ozone into the epithelial layers of the respiratory tract, ozone may only directly react with these biological antioxidant molecules in the extracellular space. Thus any detected effects of ozone on the bulk of pulmonary antioxidant pools may be mainly due to secondary reactive oxygen metabolites generated from ozone or to other reactive metabolites generated from the interaction of ozone with components of the surfactant and epithelial cell lining fluid (16).

In summary, currently available data indicate that the reaction of ozone or derived reactive oxygen metabolites with endogenous low-molecular weight antioxidants is a dominating factor for controlling the disposition of these oxidants in the lung. Their combined activity constitutes an effective antioxidant defense against the reactivity of ozone with biological macromolecules.

Reaction with lipids

For many years it has been clear that one of the major cellular macromolecular targets for ozone is the phospholipids constituting biomembranes. On the other hand, only now is an understanding of the complexity of these reactions in the biological setting being attained. This research has traditionally focused on stable, lipid-derived metabolites that can be assayed in body fluids of the exposed persons.

Classically, these reactions involve an initial, non-radical reaction of ozone with poly-unsaturated fatty acids (PUFA) across carbon-carbon double bonds, the yield being a 1,2,3-trioxalane which rearranges via carbonyl oxide and aldehyde intermediates to an ozonide via a Criegee reaction. This ozonide then decomposes to yield lipid peroxides or hydroperoxides (35, 36). More recently, however, evidence provided by Pryor (16) has suggested that, although ozonides are likely to break down to lipid peroxides in the lipophilic environment of biomembranes, in the aqueous environment of the airways the ozonides are likely to decompose via hydroxyhydroperoxy intermediates to yield further aldehyde products and hydrogen peroxide, which could then partake in metal-catalyzed Fenton reactions to generate hydroxyl radicals (figure 4).

Further evidence for these later reactions in the interaction of ozone with unsaturated fatty acids comes from the demonstration of hydroperoxy intermediates in the ozonation of unilamellar micelles of phospholipids (37). These studies also clearly demonstrate the ultimate accumulation of hydrogen peroxide, aldehydes, and carboxylates in the *in vitro* test system. These reactions have also been recently shown to occur to arachidonic acid in both subcellular systems and in cultures of pulmonary epithelial cells, the yield being aldehydic substances and hydrogen peroxide (38).

Thus, at least from work with *in vitro* systems, it appears that reactive, lipid-derived ozonides, peroxides and aldehydes, as well as hydrogen peroxide, all potentially play a role in ozone-induced toxicity in biological systems. It is also clear that, as new data become available as to the nature of the reaction products of ozone with lipid membranes, better biological markers of exposure will be defined. Indeed, there is definitely such a need as, hitherto, it has been difficult to detect products of the interaction of ozone with lung lipids *in vivo*. Early studies by Goheen et al (39) failed to show the oxidation of linoleate and arachidonic acid in the lungs of rats exposed to ozone for up to four weeks. However, Rabinowitz & Bassett (40) clearly demonstrated the accumulation of stable carboxylate oxidation products of ozonides and aldehydes derived from tissue oleate and arachidonate in the lungs of rats exposed acutely to ozone for 4 h. Similarly nonenal and heptanal were shown to

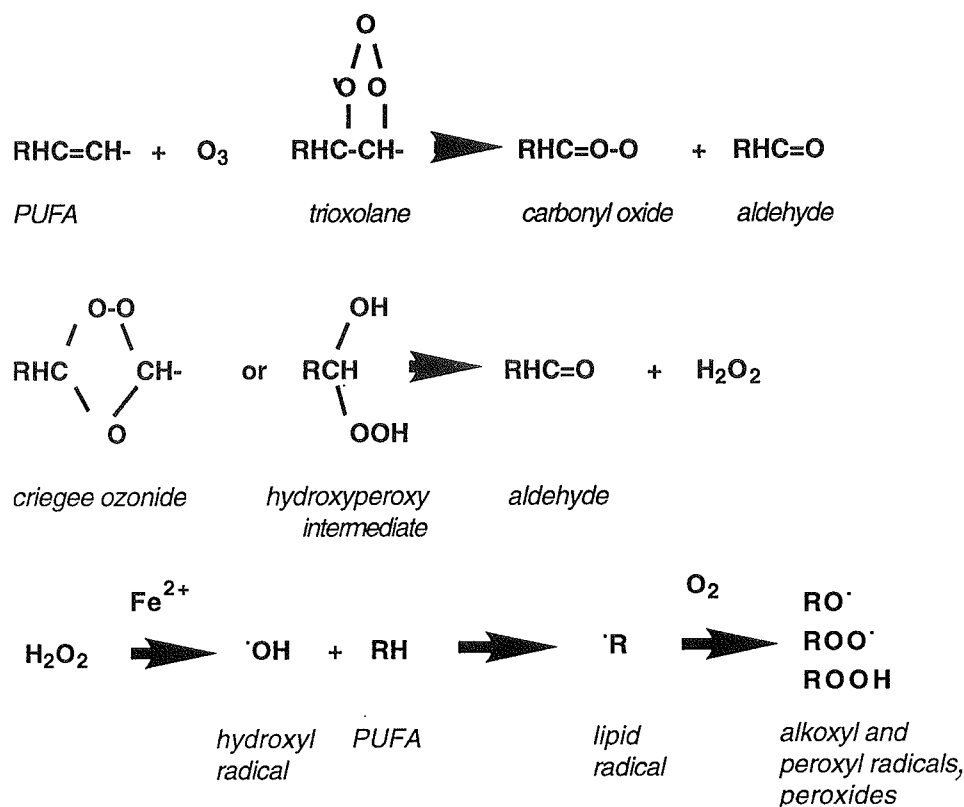


Figure 4. Major products of the interaction of ozone with polyunsaturated fatty acids (PUFA).

accumulate in the bronchoalveolar lavage fluid of animals exposed to ozone for 12 h (41). Other markers of lipid peroxidation, such as the exhalation of pentane and ethane, and the accumulation of malondialdehyde, detected as thiobarbituric acid-reactive substances in body fluids, have failed to provide convincing evidence of rife lipid peroxidation in the lungs in response to the inhalation of ozone (16, 27).

In summary, the results of in vitro experiments indicate that the reaction of ozone with the polyunsaturated fatty acids esterified in the lipids of biological membranes represents one of the major pathways of the reaction of the oxidant with biological macromolecules. Evidence is accumulating to suggest that such reactions, particularly in relation to the induction of lipid peroxidation, play a major role in the acute cytotoxicity of ozone. However, it is still uncertain if such reactions, and the production of lipid ozonides and their breakdown products, play an important role in chronic pathophysiological changes, such as the induction of inflammation, in persons exposed to ozone.

Reaction with proteins

Several amino acids in proteins are targets for direct reactions with ozone, including cysteine, methionine, tryptophane, and tyrosine (figure 5). Oxidation of these amino acids may have a variety of consequences for the

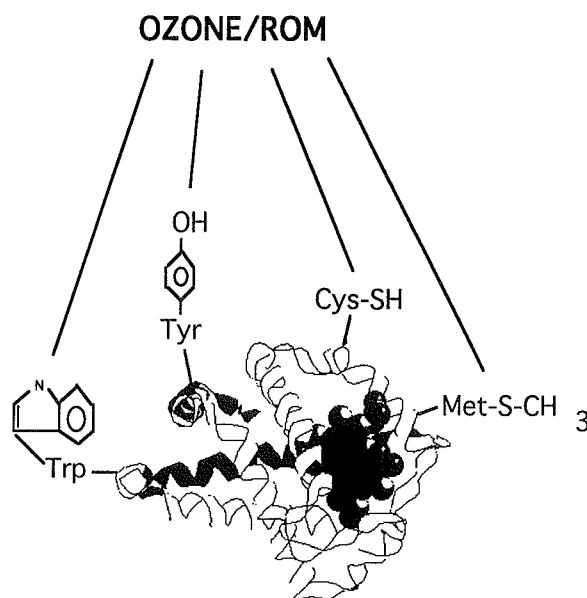


Figure 5. Amino acid targets for the reaction of ozone or reactive oxygen metabolites (ROM) or both with proteins.

function of enzymes, receptors, and other cellular proteins (42).

One of the best studied examples of such oxidation is the effects of ozone on the redox status of cysteinyl

thiols of proteins. Several studies *in vivo* have shown that the inhalation of ozone causes the oxidation of pulmonary protein thiols via the formation of mixed disulfides with glutathione (43). This reaction is accompanied by the loss of activity of several thiol-dependent enzymes in lung homogenates (44–46). It should be noted that no studies have been performed on the potential of ozone to oxidize protein thiols to higher oxidation states, such as the corresponding sulfeinic, sulfinic, and sulfonic acids. Similarly, although not directly related to thiol oxidation, many oxidant stimuli have been shown to induce the accumulation of protein carbonyls in cells and tissues (47). Such stable oxidation products might also offer promise as biomarkers of exposure, particularly in bronchoalveolar lavage samples.

Although the molecular basis of the interactions of ozone with the amino acids of proteins have, without exception, never been fully clarified, there are many specific examples of such interactions. Thus the acute exposure of animals to ozone over a few hours has been shown to inhibit P_{450} -dependent oxidation of a variety of substrates (48, 49). More recently, many studies with rodents have shown that more chronic exposures to ozone *in vivo*, over a period of days or weeks, causes the induction of P_{450} and NADPH-cytochrome P_{450} reductase in cytochromes (48, 49). This phenomenon causes a general elevation in the ability of the lung to metabolize xenobiotics, including airborne carcinogens. In addition, some of the mitochondrial enzymes that are coupled to oxidative phosphorylation have been shown to be inhibited in the lung by acute exposure to ozone *in vivo* (52, 53). The activities of other enzyme systems affected by the exposure to ozone include prostaglandin synthetase (54) and cyclooxygenase (55), which are important in the generation of important prostaglandin and leukotriene mediators involved in the induction and maintenance of inflammation. Finally, ozone has been shown to alter readily the structure and function of rodent pulmonary surfactant protein A *in vivo* (56), presumably by an oxidative mechanism involving the oxidation of critical methionine and tryptophane residues in the protein through the mediation of ozone or hydrogen peroxide (57).

In summary, although model studies with purified proteins clearly indicate that ozone reacts with several chemically reactive groups in the constitutive amino acids of a wide variety of proteins, it should be noted that there have been only few reports on the effect of exposure to ozone on human pulmonary proteins, particularly during the inhalation of the gas. Thus the only clear example is the fact that ozone effectively oxidizes and inhibits the human α -1-anti-trypsinase inhibitor (58). Clearly, further work is indicated to determine the extent of the chemical interaction of ozone with other human pulmonary proteins.

Reactions with nucleic acids

Many studies have suggested that ozone is genotoxic, possessing both mutagenicity and carcinogenicity in mammalian systems (59). It should be remembered, however, that genotoxicity can also be envisaged in terms of altered regulation of the expression of genetic information (ie, in terms of altered "gene dosage").

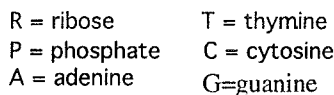
By definition, one of the central components in these processes is the ability of ozone to interact with DNA. Early studies with purine and pyrimidine bases, nucleotides and nucleotides, as well as with nucleic acids, all showed that ozone is capable of directly degrading these important materials (60). Since then, many studies have shown that ozone, or ozone-derived hydroxyl radicals, can oxidize and disturb the complex nature of nucleic acids at a variety of levels.

Thus ozonation of the double-stranded plasmid DNA was unequivocally shown to cause single- and double-strand breaks, as well as a loss of structural integrity of the plasmid by linearization, with concomitant loss of transforming action (61, 62). Similarly, in intact cells, single-strand breaks, interstrand cross-links and cross-links between DNA and protein have all been noted in response to ozone exposure *in vitro* (figure 6) (63). It should be remembered, however, that these experiments are often performed at irrelevantly high concentrations of ozone and that the systems often negate any effect of the normal cellular DNA repair mechanisms. Similarly, little attention has been paid to the potential interactions of ozone with RNA (ribonucleic acid) species (figure 6).

Thus ozone or reactive oxygen metabolites can cause a variety of alterations in the structure of DNA, including, from left to right in figure 6, double-strand breaks, sugar peroxidation, base hydroxylation, single-strand breaks, and protein-base cross-links.

Many questions remain to be answered as to the mechanisms and consequences of the interaction of ozone with cellular nucleic acids. From the point of view of potential biomarkers of exposure, further studies are required to determine the chemical nature of the interaction of ozone with nucleic acids. It would be of particular interest to see if the exposure of DNA to ozone in chemical, cellular, and *in vivo* systems induces the accumulation of hydroxylated DNA bases and products of sugar oxidation in the system, as has been proposed for DNA damage mediated through the hydroxyl radical (64). Thus the urinary excretion of 8-OH guanosine has been proposed as a marker of whole-body oxidative stress in humans (65).

It is, at present, unclear whether the interaction of ozone with DNA demonstrates some specificity. It remains to be determined, for instance, if the patterns of mutation induced by ozone in cellular DNA are entirely random.



In summary, nucleic acids present attractive sites of reaction with ozone. Current knowledge of such interactions is largely limited to DNA-damaging effects at unrealistically high concentrations in *in vitro* systems. See the section on genotoxicity and carcinogenicity in this issue (66). However, in view of the mutagenic potential of ozone and its debated ability to induce cancer, the ability of ozone to react with cellular nucleic acids, such as DNA, must receive more attention in future studies.

In this section selected examples are used to illustrate the serious perturbations in integrated biochemical processes due to the reactivity of ozone with, and the alteration of the structure of, lipids, proteins and nucleic acids. It is hoped that this section will provide a tangible bridge from events at the molecular level to the expression of alterations in the cellular phenotypes, cytotoxicity, and genotoxicity, which have been detected in the lung morphologically, cytologically, and histologically after the inhalation of ozone.

Two consequences of the oxidation of phospholipids on the biochemical functionality of membranes can be considered. Experiments using isolated perfused rat lung obtained from animals exposed to ozone over a period of 3 d clearly demonstrated increased synthesis of free fatty acids, phosphatidic acid, phosphatidyl choline, and sphin-

These changes would constitute important components of the alterations in transepithelial permeability, which is characteristic of the inflammatory state instigated by ozone in the lung (69, 70). Thus changes in membrane permeability and fluidity have been detected in alveolar macrophages obtained from ozone-exposed animals (71, 72). Short-term exposure to ozone in vivo has even been shown to affect the integrity of intracellular membranes such as those present in the mitochondria of pulmonary cells (53). In addition, ozone has been shown to increase the permeability of macromolecules in either direction between the interstitium and the airways. Thus ozone exposure increases the albumin and immunoglobulin G (IgG) content of the bronchoalveolar lavage fluid of exposed animals (73). Similarly, ozone exposure increases the uptake of instilled neutral red from the airways into the pulmonary interstitium and circulation (74).

As has already been mentioned, short- and long-term exposure to ozone instigates changes in membrane ar-

chidonate metabolism by both stimulating lipid peroxidation and changing the relative proportion of esterified arachidonic acid in membranes. These alterations to this particular component of biological membranes have important ramifications for many biochemical processes, particularly those involved in inflammation. Thus arachidonic acid metabolites, particularly those generated by 5'-lipoxygenase (leukotrienes and thromboxanes) and cyclooxygenase (prostaglandins), have been implicated in changes in airway permeability, in stimulating the infiltration, or chemotaxis, of granulocytes and lymphocytes into the lung, and in stimulating smooth muscle hyperactivity in airways.

Many studies have shown that lung cells, particularly alveolar macrophages, release elevated amounts of eicosanoids, including PGE₂ (prostaglandin E₂) (75, 76) and PGF_{2α} (prostaglandin F_{2α}) (75), thromboxane B₂, 15-HETE, LTB₄ (leukotriene B₄) and LTD₄ (leukotriene D₄) when exposed to ozone in vitro or in vivo (76). Similar effects have also been demonstrated in vitro in epithelial cells of airways (77). These results have been interpreted both in terms of an increased availability of arachidonic acid through lipid peroxidation or specific, oxidant-stimulated release mechanisms and stimulation of the synthesis of the relevant enzymes themselves. Similarly, eicosanoids, particularly various prostaglandins, have been shown to be elevated in the bronchoalveolar lavage fluid (78, 79) and plasma (80, 81) of ozone-exposed animals. It should be noted, however, that there have been few studies attempting to test the effect of ozone on the release of eicosanoids from either human alveolar macrophages or bronchial epithelial cells and their potential accumulation in bronchoalveolar lavage fluid. See the section on animal studies of ozone toxicity (82).

In summary it is clear that ozone interacts with lipid components of biological systems and generally results in stimulated degradation of membrane integrity. This process has both structural and functional implications for the viability of affected cells. Despite the ample evidence from in vitro experiments that uncontrolled lipid peroxidation is one of the primary effects of the interaction of ozone with biomembranes, evidence for this in vivo is not conclusive. However, more subtle effects (eg, the release of arachidonic acid metabolites), which have been amply described in animal studies, may be more relevant to the mechanism of the toxicity of the gas in humans. Nevertheless, it is not currently positive whether stimulated eicosanoid metabolism plays a role in ozone-induced pulmonary toxicity in humans.

Protein-dependent processes

It is difficult to present one single, representative example of how ozone-induced damage to protein affects a specific biochemical process that may be critical in the expression of the toxicity of the gas. Proteins are in-

involved in most processes in cells and, as has already been shown, it is clear that ozone, and the reactive species generated from it, oxidizes many functional groups of proteins and results in potential changes in function. It is perhaps because of this potential plethora of simultaneous events that little work has been done to identify potentially critical protein ozone "targets," which might then be useful in explaining the molecular mechanism of ozone toxicity.

In addition to causing disturbances in lung surfactant lipid structure, ozone also causes the oxidation of the surfactant protein A. This collagen-like protein constitutes 3% of the surfactant and is thought to have a variety of functions, including control of transepithelial absorption and macromolecule secretion, particularly of phospholipid components of the surfactant. The protein also stimulates various macrophage functions, including phagocytosis and the release of oxidants (83). Ozone alters the physicochemical properties of the molecule and causes an impairment of self-association and association with phospholipid vesicles (56), as well as an impaired ability to interact with alveolar macrophages (57). These effects can thus contribute to an impaired functional integrity of the protective surfactant layer of the lung. It remains to be seen if this phenomenon allows for an increased penetration of ozone and other airborne materials into the pulmonary epithelium.

One of the only other groups of human proteins which have been studied is the tear proteins secreted by lachrymal glands into tear fluid. Schmut et al (84) showed that the exposure of human tears from healthy volunteers to ozone in vitro caused rapid and extensive degradation of the tear proteins. This phenomenon may present ramifications for the maintenance of ocular function and the occurrence of eye infections during exposure to the gas.

The acute exposure of animals to ozone has been shown to affect mitochondrial function in pulmonary tissues. Thus the coupling of mitochondrial oxidative phosphorylation has been found to be reduced dramatically in pulmonary mitochondria after acute ozone exposure in vivo (52, 53). The authors proposed that this phenomenon was due to both stimulated lipid peroxidation in the inner and outer mitochondrial membranes and the oxidation of pyridine nucleotides and mitochondrial thiols. It is well established that many of the critical proteins involved in the generation of adenosine-5'-triphosphate via oxidative phosphorylation are thiol-containing and thus are sensitive to redox changes (85). Although these early studies were never followed up, particularly with respect to the identification of the proteins targeted by ozone, the example clearly illustrates how protein modification induced by ozone can seriously compromise a pivotal biochemical process supplying energy equivalents to many other cellular processes.

One area of protein research that has been totally neglected is the possibility that ozone directly or indirectly alters the redox status of receptors or signal transduction systems in pulmonary cells. A growing number of protein receptors has been shown to contain critical thiol groups whose redox status controls the reception and transduction of extracellular hormonal, growth factor, and other stimuli (86).

In addition to the oxidative modifications of proteins already presented, there is a strong possibility that aldehydic products derived from lipid peroxidation induced by ozone or reactive oxygen metabolites can interact with cellular proteins. For instance, it is well known that certain aldehydes interact directly with irritant receptors in the airways once inhaled. These reactions lead to bronchoconstriction, which may help to facilitate the development of inflammation in the airways.

In summary, the multiplicity of potential sites of reaction between ozone and proteins, coupled to the variation in cellular protein structure and the intricacy of their functional integration into biochemical processes, indicates the potential for ozone-induced alterations to protein structure and function. With the increasing availability of molecular biological and immunologic tools, it should prove possible to detect ozone-induced oxidation of proteins with increasing sensitivity and selectivity and clarify the role of such changes in the pulmonary toxicity of the gas.

DNA-dependent processes

It is clear that ozone causes alterations in the expression of normal genetic programming and ultimately leads to cell transformation. See the section on mutagenicity and carcinogenicity of ozone in this issue (66). By definition, changes in the structure of genomic DNA or the expression of genetic information must underlie these processes to a great extent. Despite the demonstration of mutagenicity for ozone (59), there are no studies reported in the literature defining whether the pattern of ozone-induced mutations is random or whether some selectivity is expressed. In addition, there have been few attempts to determine ozone-induced mutations in genes relevant to the control of cell cycling and differentiation (ie, protooncogenes and tumor suppressor genes). However, a very recent observation indicates that life-time exposure of BC63f1 mice to ozone significantly increases the frequency mutation of the K-ras protooncogene in lung neoplasms (87).

In addition to relying on a mutational basis, DNA-dependent toxicity can also be defined in terms of altered levels of expression for native genes and thus it alters normal patterns of gene "dosage." A few studies have shown the induction of the expression of specific cellular genes in response to exposure to ozone. For instance, the levels of transcription of antioxidant enzymes such as

glutathione peroxidase, catalase and superoxide dismutase (32), various cytochromes P₄₅₀ (51), a variety of pulmonary extracellular matrix proteins, including fibronectin (88) and collagen (89), various macrophage proinflammatory proteins (90, 91), and an, as yet, uncharacterized gene coding for a 45KDa substrate from bronchial epithelial cells (92) are all altered in response to ozone.

It is uncertain whether ozone directly interacts with the respective genes to change the rate and extent of expression or whether the interaction occurs at some other level of the transcriptional or translational regulation. For instance, it is becoming increasingly clear that the activity of many mammalian transcription factors, proteins which bind to specific regions of promoters upstream from the coding region of genes, may be regulated by redox changes to the proteins themselves (93). In addition, it may be that metabolites derived from the interaction of ozone with biomolecules are involved in the execution of these induction events.

In summary, it is becoming clear that many of the phenotypic changes seen within different cell populations in the lung in response to the inhalation of ozone are, in the long-term, controlled by stimulated alterations to gene expression. Irrespective of the mechanisms involved, further work may well identify specific gene inductions that may provide sensitive biomarkers for early exposure to ozone. In this respect, analysis of the transcription rates of various proinflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukin-1 (IL-1), IL-6 and IL-8 in cells of bronchoalveolar lavage or peripheral blood might provide useful tools. Indeed, recent studies of both human pulmonary epithelial cells and macrophages exposed to ozone *in vitro* have shown induction of the expression of all of the aforementioned inflammatory cytokines from macrophages (94, 95), while others have suggested that epithelial cells may be the primary source of ozone-stimulated IL-6 and IL-8 release in the lung (94).

Summary and concluding remarks

It is clear that the chemical reactivity of ozone and its rapid degradation to reactive oxygen metabolites, such as the hydroxyl radical, at biologically relevant pHs, facilitate a reaction with most classes of biological macromolecules. Similarly, this reactivity, in all probability, restricts the existence of ozone itself to the lumen of the lung after the gas is inhaled. These concepts present serious problems with respect to efforts to define the critical events leading to the pulmonary toxicity of ozone at the molecular level. Many reactions are possible. However, it is clear that reactions with polyunsaturated fatty

acids in biological membranes, the induction of lipid peroxidation, and the release of biologically active metabolites such as arachidonic acid may play a role in both the acute cytolytic toxicity of the gas and the development of chronic toxicity, such as the induction of inflammation. On the other hand, less is known of the potential importance of the interaction of ozone with other cellular macromolecules, particularly with respect to the oxidation of proteins and DNA and the consequences of these events for the long-term maintenance of cellular phenotype by adaptation within the lung.

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