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Combined Exposure to Ozone and Nitrogen Dioxide

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The combined effects of ozone and nitrogen dioxide were assessed in an *in vitro* test system utilizing human red cells. In general, these two pollutants had additive effects on the parameters measured which included osmotic fragility, acetylcholinesterase activity, *lipid peroxidation, reduced glutathione and methemoglobin levels. However, at lower* pollutant doses a synergistic increase in lipid peroxides was noted while at higher doses the effect became less than additive. Further studies of this observation suggested that ferrous hemoglobin potentiates ozone-induced lipid peroxidation while methemoglobin, resulting primarily from nitrogen dioxide, inhibits this process.

Ozone was also found to potentiate the methemoglobinemic effects of nitrogen dioxide, particularly in sequential studies in which ozone exposure preceded nitrogen dioxide.

Inasmuch as the effects of these two pollutants vary from protective to synergistic depending on the pollutant concentration, duration and sequence of exposure, as well as on the parameter assayed, it would appear that the approach to *in vivo* study of the combined effects of ozone and nitrogen dioxide should be aimed at simulating ambient conditions as *closely as possible.*

Study of the effects of multiple pollutants inhaled simultaneously is a relatively unexplored area of air pollution research, despite the fact that it is recognized that the toxicity of such mixtures may not be predictable on the basis of response to the individual components (*1*). The lack of information is due both to the complexities in performing such studies and, at least in the United States, to the focus on individual air pollutants inherent in the Clean Air Act of 1970.

The present studies consist of our initial evaluation of the combined effects of ozone and nitrogen dioxide. There are a number of similarities in the toxic effects of these pollutants which are both present in significant concentrations in urban areas where photochemical smog occurs. At high concentrations both ozone and nitrogen dioxide produce death in pulmonary edema. Tolerance to lethal levels following exposure to sublethal concentrations occurs with each, as does cross-tolerance to the effect of the other (*2,3*). At relatively low levels both have been shown to produce animal lung lipid peroxidation (*4–6*). In concentrations approaching air quality standards, both ozone and nitrogen dioxide potentiate respiratory infections in animals and for each it appears that their major effect on alveolar macrophages is interference with intracellular killing of bacteria (*7–9*). Long-term exposure to either pollutant results in pathological changes suggestive of chronic respiratory disease (*10,11*).

It is, of course, recognized that substantial differences in the toxicity of these two pollutants do exist. Ozone is a far more powerful oxidant, while nitrogen dioxide is an acid anhydride and conceivably exerts part of its toxicity through the formation of nitrites. There are both similarities and distinct differences in animal lung pathology. It is conceivable that both the differences and the similarities in their toxic effects at the cellular level may play a role in determining the degree to which interactions between these two pollutants occur in the human lung. Previous studies of the combined

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effects of ozone and nitrogen dioxide include the suggestion of additive pathological damage in the lungs of rats (*12*) and the report of indifferent effects on the bacteriocidal function of mouse alveolar macrophages (*13*).

Our initial studies of the combined effect of ozone and nitrogen dioxide have been performed in an *in vitro* model system previously used in this laboratory for the study of ozone toxicity. The rationale for the use of an *in vitro* model is both to evaluate potential parameters for future animal studies and to ascertain whether such a model might be useful in predicting the *in vivo* effects of combined exposure. The model consists of washed human red cells suspended in phosphate buffered (0.01*M*) saline, pH 7.4. The red cells are exposed to ozone and nitrogen dioxide in specially fabricated double-inlet fritted disc bubblers in which mixing of the pollutants occurs only within the red cell suspension. In most of these studies four bubblers are used, one of which receives both ozone and nitrogen dioxide. The ozone and nitrogen dioxide streams are split in two, with one half of the ozone stream going to the second fritted disc bubbler and nitrogen dioxide to the third. Both of these bubblers also receive an input of filtered room air while the fourth bubbler is gassed with filtered room air through both bubblers.

The parameters chosen for study include osmotic fragility (*14*), an indicator of red cell membrane integrity; malonaldehyde (MDA) formation (*15*), a parameter of cell membrane lipid peroxidation; the activity of acetylcholinesterase (AChE) (*16*), a cell membrane enzyme whose activity decreases in association with a number of free radical and oxidative membrane processes (*17,18*); reduced glutathione (GSH) (*19*), an intracellular tripeptide which functions as a scavenger of free radicals and peroxides; and the formation of methemoglobin (metHb) (*20*), the ferric form of hemoglobin.

Data have been obtained at a number of different concentrations of ozone and nitrogen dioxide. Four representative experiments are shown in Table 1. In general, the data appear to show generally additive effects. However, two anomalies are apparent. The first is that at lower concentrations of ozone and nitrogen dioxide there tends to be a more than additive increase in lipid peroxides, measured as malonaldehyde formation, while at higher concentrations of these two pollutants a less than additive effect occurs. This is more clearly demonstrated in Figure 1, where samples obtained during the initial period of combined exposure to ozone and nitrogen appear to show a synergistic effect. In contradistinction, during the latter part of the exposure period less MDA is present than would be expected from the individual action of these pollutants. Further information was obtained in sequential studies in which the effect of exposing red cells to first one pollutant and then the other is compared to simultaneous and individual exposure. Sequential exposure in any order to low concentrations of ozone and nitrogen

Table 1. Effects of combined and individual *in vitro* exposure to ozone and nitrogen dioxide on human red cell osmotic fragility, malonaldehyde (MDA), acetylcholinesterase (AChE) activity, glutathione (GSH), and methemoglobin.[a]

Ozone, ppm	Nitrogen dioxide, ppm	Osmotic fragility (50% hemolysis), g/100 ml NaCl	MDA, nmole/g Hb	AChE, % of Control	GSH, % of control	MetHb, %
2.	20	0.48	18	80	84	79.5
2	0	0.47	10	84	89	2.2
0	20	0.44	4	99	95	72.6
0	0	0.43	0	100	100	0.4
2.2	3.6	0.45	16	80	82	18.8
2.2	0	0.44	11	86	84	2.1
0	3.6	0.43	2	98	97	14.0
0	0	0.42	0	100	100	0.6
38	4.1	0.66	46	30	36	24.0
38	0	0.66	42	38	38	4.6
0	4.1	0.44	2	98	96	17.1
0	0	0.43	0	100	100	0.3
41	102	0.64	32	32	14	100
41	0	0.66	40	34	28	6.8
0	102	0.45	11	95	86	100
0	0	0.41	0	100	100	0.3

[a]The red cells were analyzed after 2 hr of exposure. All assays were performed in duplicate.

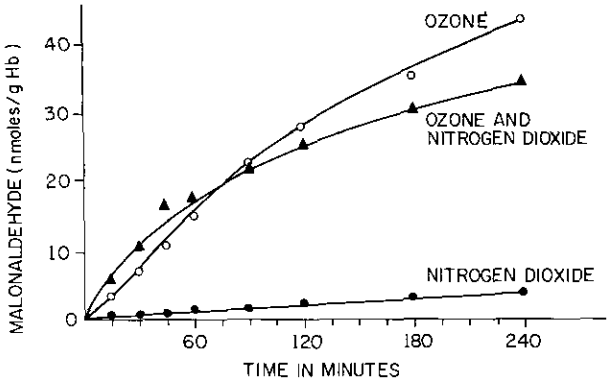

FIGURE 1. Malonaldehyde formation in red cell suspensions exposed to (o) 10.2 ppm ozone, (●) 32 ppm nitrogen dioxide, or (▲) to both simultaneously.

dioxide resulted in generally additive rather than synergistic effects on lipid peroxidation. At higher concentrations additive effects were also observed when ozone preceded nitrogen dioxide exposure. However, when ozone was given following exposure to nitrogen dioxide concentrations producing appreciable methemoglobin formation, there was a decrease in the expected levels of MDA. Furthermore, preincubation of red cells with sodium nitrite so as to produce 100% methemoglobin levels also resulted in lesser MDA formation upon subsequent ozone exposure.

No effect of nitrite was observed on the thiobarbituric acid assay system used to measure MDA, nor was the protective effect of high levels of nitrogen dioxide observed when red cell membranes were substituted for intact red cells in the in vitro exposure model. Based on these studies we have tentatively concluded that oxygenated ferrous hemoglobin (oxyhemoglobin) potentiates ozone-induced lipid peroxidation and that the formation of methemoglobin by nitrogen dioxide therefore interfered with this reaction. While the pertinence of these *in vitro* studies is questionable, it should be noted that in cities such as Los Angeles where the automobile is the main source of nitrogen dioxide, peak nitrogen dioxide values tend to precede peak ozone values by 1 or 2 hr.

A second area of interest in the data is the apparent potentiation by ozone of the methemoglobinemic effects of nitrogen dioxide. In sequential studies this was most apparent when ozone preceded nitrogen dioxide exposure. The biochemical basis for this observation has not been elucidated. However, it has been noted that red cells previously exposed to ozone and nitrogen dioxide have a lesser decrease in methemoglobin levels following overnight incubation with glucose than do red cells exposed to nitrogen dioxide alone. This would suggest that ozone interferes with the normal metabolic processes resulting in the reduction of methemoglobin to ferrous hemoglobin, perhaps by inactivation of the enzyme NADH-methemoglobin reductase, or by interfering with the availability of NADH.

The present findings illustrate the potential complexities involved in the assessment of the effects of combined exposure to two pollutants. The data clearly indicate that for these two pollutants and for this *in vitro* system the absolute and relative concentration of pollutants as well as the time course and sequence of administration will affect the expression of toxic interactions.

It must be emphasized that the applicability of these *in vitro* findings to humans breathing both of these pollutants is not known, nor can it be assumed that other combinations of pollutants would be affected by similar variables either *in vitro* or *in vivo*. However, the results do suggest that animal inhalation experiments assessing the combined effects of ozone and nitrogen dioxide should be performed at various concentrations, time courses, and sequences of exposure. Furthermore, if animal experiments do indicate that such variables affect the toxic interaction of pollutants, these findings would question the validity of assessing combined exposure by a methodological approach that utilizes higher than expected ambient concentrations of pollutants and then assumes that the same dose–response relationship will be present at ambient concentrations.

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