Distinct Enzymatic Responses in Mice Exposed to a Range of Low Doses of Ozone

by T. S. Veninga,* J. Wagenaar,* and W. Lemstra*

Short-term exposure of mice to low O₃ doses, as defined by the product of concentration and exposure time (ct), was observed to induce alterations in two enzyme systems: first, that leading to changes in hepatic reduced ascorbic acid (RAA) content, and second to changes in plasma creatine phosphokinase (CPK) activity. RAA alterations were noticed immediately, 30 min and 120 min after termination of the exposure period, whereas CPK showed alterations immediately and 15 min after termination of the exposure. Later determinations, i.e., 24 hr after O₃ exposure for RAA and 30 min after exposure for CPK, revealed no significant differences when compared to control animals. Although differences in sensitivity existed, the dose response curves for both systems were more or less similar, showing a short decrease for the initial very low O₃ doses, followed by a profound rise and a gradual decrease to control levels for subsequent ct doses. Exceptions were the 30 min curve for RAA and the immediate curve for CPK in so far as both showed an additional depression.

Neither plasma histamine nor plasma lactic acid dehydrogenase (LDH₃) were observed to be altered by the range of O₃ doses employed. These findings were explained on the basis of adaptation of the organism to a potentially noxious O₃ stimulus by enhanced metabolic processes: a weak stimulus leading to only a small adjustment, and stronger stimuli to elevated enzyme activity as well. With increasing doses of O₃ this elevation in enzyme activity was found to be gradually diminished, possibly due to a steadily growing demand, leaving the overshoot becoming continually smaller until a balanced state is achieved.

Radiomimetic activity of ozone (O₃), first described by Brinkman and Lamberst (1) and supported by the suggestion of Goldstein et al. (2-4) that O₃ action in living organisms may be mediated by the formation of free radicals, might have led investigators to consider every O₃ action as being harmful regardless of the O₃ concentration and the duration of exposure (5, 6). In the opinion of these authors there is no threshold value (no-effect level), and the dose-effect curve starts at zero. However, in the same way as with ionizing irradiation, in order to visualize effects, dosages of O₃ have to be explored which in general clearly exceed the permissible value for industrial workers (i.e., 0.1 ppm/8 hr = 200 μg/m³/8 hr).

Exposure times of days or weeks have often been used under experimental conditions. There is no doubt that, in the higher concentration time ranges, O₃ causes toxic symptoms. The linearity of the dose-effect relationship of hazardous O₃ action, down to zero, must be disputed, as it has been for ionizing irradiation (7). Even more so, since nonanthropogenic O₃ is a natural ambient constituent for which an adaptive mechanism to lower values might be supposed to be present. Currently, such a mechanism has actually been suggested in the results of a number of studies, including those done in man (8-11).

The results of the present study, where mice are exposed to O₃ doses in a range regularly occurring in ambient air, are in favor of the existence of such an adaptive process.

---

*Laboratory for Radiopathology, State University of Groningen, Bloemsingel 1, 9713 BZ Groningen, The Netherlands.
Materials and Methods

Groups of 15-20 male mice of an inbred grey strain (Gron), as well as of the hybrid of this strain with inbred C57Bl mice (F1) were exposed to O3, with five mice to a cage. Similar groups of mice were concurrently sham-exposed. Cages were placed in a stainless steel cabinet. The animals had full access to water, but they were deprived of food for 16 hr prior to and in the period of O3 exposure. O3 was generated by an adjustable silent-discharge ozonizer (Air-co Swiss) situated at the bottom of the cabinet.

The oxidant was monitored by a Dasibi type 1003AH ozonometer (Dasibi, USA) operating on the principle of UV absorption by O3. Blood for investigation was drawn from the orbital sinus under light ether anaesthesia.

Reduced ascorbic acid (RAA) in the liver was determined as previously described (12). Creatine phosphokinase (CPK) in blood plasma was assayed with the CPK test-combinations of Boehringer-Mannheim (W. Germany). Plasma histamine was determined according to the single-isotope method described by Beaven et al. (18), with some minor modifications. In our study, the sensitivity of this method was approximately 1 ng/ml. Total lactic acid dehydrogenase (LDH) in blood plasma was assessed with the LDH test-combinations of Boehringer-Mannheim (W. Germany). LDH isoenzymes were separated by disc gel electrophoresis (14), and the gels quantitatively scanned by means of a Gilford model 2400 S automatic spectrophotometer with linear transport equipment operating at 500 nm (Gilford, Ohio, USA). Results were expressed in percentages of those found in the controls and plotted against the product of concentration (c) and exposure time (t). As has been demonstrated earlier, when c and t are adapted so that the product ct remains constant, identical results are obtained (15). Student's t-test and a test based on normal approximation were used for statistical evaluation of the results. The level of significance was set at 5%. The standard error of the percentage difference between experimental and control values at each ct value was calculated as 100 times the square root of the expression:

\[
\frac{(SE_2)^2}{(\bar{X}_1)^2} + \frac{(SE_1)^2}{(\bar{X}_2)^2}
\]

where SE1 = standard error of the mean of control values, SE2 = standard error of the mean of experimental values, \( \bar{X}_1 \) = mean of control data, and \( \bar{X}_2 \) = mean of experimental data.

Results

The data obtained for hepatic RAA for three different post-exposure time intervals, i.e., for 0, 30, and 120 min, are plotted in Figure 1. Each of the three curves starts with a short negative period, followed by a sharp rise to values significantly above zero. Thereafter the curves level off to normal at the higher ct values tested. A few measurements performed 24 hr after O3 exposure reveal RAA values falling within the normal range.

\[
\begin{align*}
&20 \text{ per cent of control} \\
&\text{04} \quad \text{08} \quad \text{12} \quad \text{16} \quad \text{20} \quad \text{24} \quad \text{28} \quad \text{32} \\
&\text{ct (ppm hr)}
\end{align*}
\]

Figure 1. Reduced ascorbic acid (RAA) content of murine liver tissue expressed as a percentage of control values at three different time intervals after exposure of the animals to various concentrations of ozone (O3) for various time periods (ct); (c) immediately after exposure; (O 3) 30 min after exposure; (O) 120 min after exposure. Each point is an estimate based on the results from at least 40 experimental animals and 40 controls. The vertical bars at each point represent the standard error of this estimate. Maximal O3 concentration 1600 μg/m3; maximal exposure time 4 hr.

Similar curves were obtained for plasma CPK immediately and 15 min after O3 exposure (Fig. 2). This enzyme appears to be more sensitive, in that lower ct values are capable of evoking alterations in the enzyme activity. Furthermore, the effect of O3 on CPK disappears more rapidly; 30 min after termination of the O3 supply, significant alterations were no longer observed. Values obtained 15 min after O3 exposure also failed to show significant changes, with the exception of the CPK value obtained at ct = 0.8.

The histamine content of blood plasma remained unaltered after treatment of mice with a series of O3 concentrations for various time periods (Table 1). In addition, both total LDH as well as its isoenzyme remaining within normal limits (Table 2).

Discussion

Since RAA is a product of a chain of enzymatic activities, and since O3-induced changes in RAA are of an enzymatic nature (12), we have in fact...
Figure 2. Creatine phosphokinase (CPK) activity of murine blood plasma expressed as a percentage of control values at two different time intervals after exposure of the animals to various concentrations of ozone (O₃) for various time periods (ct): (●) immediately after exposure; (×) 15 min after exposure. Each point is an estimate based on the results from at least 30 experimental animals and 30 controls. The vertical bars at each point represent the standard error of this estimate. Maximal O₃ concentration 1600 µg/m³; maximal exposure time 4 hr.

recorded alterations in two enzymatic systems in this study.

Although differences in sensitivity between the two systems occur, the alterations are principally similar. They are restricted to the lower O₃ doses tested. This, together with the rapid reversibility of the effects may indicate that we are dealing with a physiological phenomenon rather than with cellular injury. In this sense, the initial negative part of the curves obtained might just represent a certain metabolic adjustment due to weak O₃ action. Subsequent higher doses may stimulate enzyme activity (an all-or-none effect) (12) in concert with increasing consumption until a new equilibrium may be achieved where experimental and control values are similar. Such a course with increasing O₃ doses might be characterized as adaptive and noninjurious. This is supported by the finding that plasma histamine and LDH₃ are not significantly changed. A decrease in histamine has been demonstrated in the lungs of mice and rats exposed to oxidants (16-18), whereas increased levels of plasma LDH₃ are suggested to be indicative of lung tissue damage (19).

The use of ct values seems justified under the conditions employed in this study, where low O₃ concentrations and short exposure times are combined. The method has been validated for toxicological research (20) and has also been used in air-pollutant investigations (21). We have also verified this method for some enzymes using a single ct product with different c and t values and the product was found to be constant (15, 22).

The sensitivity of different enzyme systems in reacting to an adverse stimulus appears to be different. At least, lower ct doses are capable of inducing alterations in plasma CPK compared with those leading to modified RAA values of the liver.

Other enzymes have been observed to show altered activity after O₃ exposure, for example, acetylcholinesterase (Ach-ase) activity is decreased in the erythrocytes of mice after exposure to 8 ppm O₃ for 4 hr (3).

Increases in rat lung glutathione peroxidase (GSHP), glutathione reductase (GSHR) and glucose-6-phosphate dehydrogenase (G-6-PDH) have been observed by Chow and colleagues (5, 23-25) after several doses of O₃, the lowest dose being 4 ppm for 8 hr.

G-6-PDH as well as LDH are enhanced, whereas Ach-ase is lowered in human erythrocytes after exposure to 0.5 ppm O₃ for 2.75 hr. Serum GSHR is concomitantly decreased (8).

Plasma glutamate pyruvate transaminase (SGPT) rises in mice treated with 0.2 ppm O₃ for 2 hr (12).

Ishiwatari (26) has also found an increase in pulmonary GSHP in rabbits and mice exposed to at least 5 ppm O₃ for 4 hr. Glucose-6-phosphatase is concurrently lowered in murine lungs, whereas

| Exposure | Mean plasma histamine ± SEM, µg/m³ (no. of animals) |
|----------|----------------------------------------------------|
| Time, hr | O₃ concn, µg/m³ | Immediately | After 2 hr |
|          | Controls | Exposed           | Controls | Exposed |
| 2        | 100      | 15.0 ± 1.9 (21)   | 19.0 ± 2.1 (20) | 11.2 ± 1.5 (9) | 11.6 ± 1.5 (11) |
| 2        | 140      | 8.6 ± 0.6 (10)    | 9.1 ± 0.9 (12) | 13.9 ± 1.0 (37) | 14.5 ± 1.0 (44) |
| 2        | 400      | 24.6 ± 1.4 (16)   | 23.1 ± 2.7 (23) | 21.0 ± 2.9 (11) | 18.6 ± 2.0 (13) |
| 2        | 1600     | 26.8 ± 3.9 (20)   | 25.9 ± 3.7 (22) | 24.6 ± 3.5 (18) | 22.0 ± 2.8 (24) |

June 1981
ATP-ase shows lower values in the lungs of rabbits exposed to 10 ppm $O_3$ for 2 hr per day on five successive days.

Lee et al. (27) have treated rats with 0.2, 0.5, and 0.8 ppm $O_3$ for 1 to 30 days and obtained elevated levels of lung succinate oxidase, cytochrome-c reductase and also G-6-PDH.

In man the values for Ach-ase, G-6-PDH and phosphokinase in red blood cells are unaltered after exposure to 0.2 ppm during a normal working day, but LDH and $\alpha$-hydroxybutyrate dehydrogenase levels decrease (25).

In most cases cited above, the $O_3$ doses used were significantly higher than those for which we observed altered enzyme activity, but the reversibility of the reaction has not been considered. Although elevated blood levels of some enzymes are indicative of tissue injury, and most authors tend to explain their findings to be those of an injurious nature, one may question whether the possibility of adaptation has been sufficiently taken into account, as was done by Buckley et al. (8) and Hackney et al. (9).

The second depression in two of our curves, at $ct = 0.8$ and at $ct = 2.0$ for CPK, determined immediately, and for RAA 30 min after $O_3$ exposure respectively, cannot be precisely interpreted. They certainly point to metabolic imbalance. In both cases later values (at 15 min and 2 hr after exposure respectively) show a positive overshoot.

Increased RAA levels have been claimed (12) to contribute to the organism's defense. CPK is involved in the generation of ATP from creatine-phosphate, and in this way supports the easily available energy source of the organism. This might also be considered to be part of the defensive potency of the organism. In fact, mobilization of defense may imply a scale of diverse reactions which will stabilize at a certain level if the noxious stimulus is not too strong. At that point the organism reaches the adaptive state.

We explored $O_3$ doses equal to or slightly higher than those occurring in environmental air. To conclude that these $O_3$ doses can be safely borne is premature, since we tested only one animal species. Moreover, $O_3$ is frequently present in combination with other pollutants and our knowledge of combined effects is fragmentary (12, 29-31). The occurrence of mutational events under influence of air pollutants is still under discussion (32, 33).

The authors express their gratitude to Dr. V. J. Fidler for his expert advice on statistics, and to Mrs. Annet van Dijk, Mrs. Anneke Smid, Mrs. Theo Oosterhoff, and Mrs. Ieteke Peeters for their skilful technical assistance.

This study was carried out with financial support from the Organization for Health Research TNO, The Netherlands (project G.O.77-3).

**REFERENCES**

1. Brinkman, R., and Lamberts, H. B. Ozone as a possible radiomimetic gas. Nature 181: 1202 (1958).
2. Goldstein, B. D., and Balchum, O. J. Effect of ozone on lipid peroxidation in the red blood cell. Proc. Soc. Exptl. Biol. Med. 126: 356 (1967).
3. Goldstein, B. D., Pearson, B., Lodi, C., Buckley, R. D., and Balchum, O. J. The effect of ozone on mouse blood in vivo. Arch. Environ. Health 16: 648 (1968).
4. Goldstein, B. D., Lodi, C., Collinson, C., and Balchum, O. J. Ozone and lipid peroxidation. Arch. Environ. Health 18: 631 (1969).
5. Chow, C. K., Dillard, C. J., and Tappel, A. L. Gluthathione peroxidase system and lysozyme in rats exposed to ozone or nitrogen dioxide. Environ. Res. 7: 311 (1974).
6. Tappel, A. L. Vitamin E and selenium-glutathione peroxi-
dase in protection against oxidant damage (Abstr.). Envi-
ron. Health Perspect. 16: 186 (1976).
7. Dickson, D. Sparks continue to fly in low-level radiation
down. Nature 290: 180 (1979).
8. Buckley, R. D., Hackney, J. D., Clark, K., and Posin, C.
Ozone and human blood. Arch. Environ. Health 30: 40 (1975).
9. Hackney, J. D., Lin, W. S., Buckley, R., and Hislop, H. J.
Studies in adaptation to ambient oxidant air pollution.
Effects of ozone exposure in Los Angeles residents vs. new arrivals. Environ. Health Perspect. 18: 141 (1976).

Environmental Health Perspectives

---

**Table 2. Mean plasma values of total LDH and LDH$_3$ determined either immediately or 15 min after exposure of mice to various concentrations of $O_3$ for various time periods as compared to simultaneously determined values for nonexposed controls.**

| Exposure | Mean plasma values of enzyme ± SEM, U/l. (no. of animals) |
|----------|----------------------------------------------------------|
| Time, hr | $O_3$ conc., µg/m$^3$ | Time of determination | Controls | Exposed | Controls | Exposed |
|----------|----------------------|-----------------------|----------|---------|----------|---------|
| 1        | 800                  | Immediate             | 212 ± 7  | 226 ± 8 | 24 ± 1   | 25 ± 1  |
| 2        | 400                  |                       | 215 ± 9  | 235 ± 8 | 26 ± 2   | 24 ± 2  |
| 4        | 2000                 |                       | 227 ± 14 | 240 ± 12| 26 ± 2   | 31 ± 2  |
| 2        | 800                  | After 15 min          | 249 ± 16 | 211 ± 14| -        | -       |
| 2        | 1200                 |                       | 277 ± 32 | 274 ± 21| 40 ± 3   | 49 ± 4  |

Mean plasma values of enzyme ± SEM, U/l. (no. of animals)
10. Hackney, J. D., Lin, W. S., Karuza, S. K., Buckley, R., Law, D. C., Bates, D. V., Hazucha, J., Pengelly, L. D., and Silverman, F. Effects of ozone exposure in Canadians and Southern Californians. Arch. Environ. Health 32: 110 (1977).

11. Zitnik, L. A., Schwartz, L. W., McQuillen, N. K., Zee, Y. C., and Osebold, J. W. Pulmonary changes induced by low-level ozone: morphological observations. J. Environ. Pathol. Toxicol. 1: 365 (1978).

12. Veninga, T. S., and Lemstra, W. Extrapolmonary effects of ozone whether in the presence of nitrogen dioxide or not. Int. Arch. Occup. Environ. Health 34: 209 (1975).

13. Beaven, M. A., Jacobsen, S., and Horáková, Z. Modification of the enzymatic isotopic assay of histamine and its application to measurement of histamine in tissues, serum and urine. Clin. Chim. Acta 37: 91 (1972).

14. Dietz, A. A., and Lubrano, T. Separation and quantitation of lactic dehydrogenase isoenzymes by disc electrophoresis. Anal. Biochem. 20: 246 (1967).

15. Veninga, T. S. Lemstra, W., and Wagenaar, J. Low level ozone induced biochemical changes in mice. Proc. 5th Int. Clean Air Congr. Buenos Aires, 1980, in press.

16. Dixon, J. R., and Maintain, J. T. Role of histamine and related substances in development of tolerance to edemagenic gases. Toxicol. Appl. Pharmacol. 7: 756 (1965).

17. Vyskočil, A., and Tušil, M. Changes in concentration of histamine in rat lungs in aspiration of nitrogen oxides. Cesko. Hyg. 23: 49 (1978).

18. Von Nieding, G., and Wagner, H. M. Effects of NO2 on chronic bronchitis. Environ. Health Perspect. 29: 137 (1979).

19. Bloor, C. M., Leroy, E. P., Friedman, P. J., and Sobel, B. E. Detection of pulmonary irradiation injury by determining plasma lactic dehydrogenase (LDH) activity. Radiat. Res. 57: 311 (1974).

20. W.H.O. Principles and methods for evaluating the toxicity of chemicals. Part 1. Environ. Health Criteria 6: 128 (1978).

21. U.S. Air Quality Criteria for Photochemical Oxidants. U.S. Department of Health Education and Welfare, National Air Pollution Control Adm., Washington, D.C., 1979.

22. Veninga, T. S., and Wagenaar, J. Reinforcement of ozone action in mice by additional environmental compounds. V.D.I. Ber. 270: 119 (1977).

23. Chow, C. K., and Tappel, A. L. An enzymatic protective mechanism against lipid peroxidation damage to lungs of ozone-exposed rats. Lipids 7: 518 (1972).

24. Chow, C. K., and Tappel, A. L. Activities of pentose shunt and glycolytic enzymes in lungs of ozone-exposed rats. Arch. Environ. Health 26: 205 (1973).

25. Chow, C. K. Biochemical responses in lungs of ozone-tolerant rats. Nature 260: 721 (1976).

26. Ishiwatari, T. Studies on biochemical influence of ozone exposure on lung tissue and trachea-bronchial system. Nihon Univ. Med. J. 18: 169 (1976).

27. Lee, S. D., Hacker, A. D., Ospital, J. J., and Mustafa, A. M. Influence of dietary antioxidants on low-level oxidant exposure. Proc. 4th International Clean Air Congress, Tokyo, 1977, pp. 20-22.

28. Sarto, F., Trevisan, A., Gasparotto, G., Rosa, A., and Fabbri, L. Study of some erythrocyte and serum enzyme activities in workers exposed to low ozone concentrations. Int. Arch. Occup. Environ. Health 43: 99 (1979).

29. Freeman, G., Juhos, L. T., Furiosi, N. J., Mussenden, R., Stephens, R. J., and Evans, M. J. Pathology of pulmonary disease from exposure to interdependent ambient gases (nitrogen dioxide and ozone). Arch. Environ. Health 29: 203 (1974).

30. Von Nieding, G., and Wagner, H. M. Experimental studies on the short-term effects of air pollutants on pulmonary function in man: two-hour exposure to NO2, O3 and SO2 alone and in combination. Proc. 4th Int. Clean Air Congr. Tokyo, 1977, pp. 5-8.

31. Last, J. A., and Cross, C. E. A new model for health effects of air pollutants: evidence for synergistic effects of mixtures of ozone and sulfuric acid aerosols on rat lungs. J. Lab. Clin. Med. 91: 328 (1978).

32. Von Nieding, G. Possible mutagenic properties and carcinogenic action of the irritant gaseous pollutants NO2, O3 and SO2. Environ. Health Perspect. 22: 91 (1978).

33. Guerrero, R. R., Rounds, D. E., Olson, R. S., and Hackney, J. D. Mutagenic effects of ozone on human cells exposed in vivo and in vitro based on sister chromatid exchange analysis. Environ. Res. 18: 336 (1979).