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# Lipid Peroxidation and Antioxidative Protection Mechanism in Rat Lungs upon Acute and Chronic Exposure to Nitrogen Dioxide

## by Masaru Sagai* and Takamichi Ichinose*

**This work was done to clarify the relation between the changes of lipid peroxidation and the activities of antioxidative protective enzymes in lungs of rats exposed acutely, subacutely, and chronically to nitrogen dioxide. It was confirmed that the activities of the antioxidative enzymes to protect cells from oxidative stress increased in an early phase, and then the activities decreased gradually. Lipid peroxides increased once in an early phase and then returned to the control level; thereafter, lipid peroxides increased gradually again. Lipid peroxidation as measured by ethane exhalation increased significantly with 0.04, 0.4, and 4 ppm nitrogen dioxide exposure for 9, 18, and 27 months, and a dose-response relationship was clearly observed. The temporal changes of lipid peroxidation varied inversely with that of the activities of antioxidative protective enzymes.**

**From these results, it was suggested that the increments of antioxidative protective enzyme activities in an early phase were complementary effects to protect cells from damage by lipid peroxides which were increased by nitrogen dioxide exposure, and that the complementary effects are lost in later phases of life-span exposure. Finally, loss of such protective complementary effects might relate to some chronic diseases in lungs. Therefore, the temporal changes described above are important characteristics in chronic exposure of air pollutants.**

## Introduction

Nitrogen dioxide ($NO_2$) is a strong oxidizing pollutant commonly found in urban air. The toxicity of $NO_2$ has been studied in a number of animal species. $NO_2$-related studies of lung biochemistry have been directed to either an investigation of the mechanism of toxic action of $NO_2$ or to the detection of early damage by $NO_2$ inhalation. Two theories of action of $NO_2$ on biological systems have evolved as a result of these studies. The dominant theory is that $NO_2$ initiates lipid peroxidation, which subsequently causes cell injury or death and the symptoms associated with $NO_2$ inhalation. The second theory is that $NO_2$ oxidizes low molecular weight reducing substances and proteins. This oxidation results in a metabolic dysfunction which evidences itself as the toxic symptom. Several potential biochemical responses to $NO_2$ intoxication have been proposed. Lipid peroxidation by $NO_2$ and the several potential biochemical defense mechanisms against $NO_2$ will be discussed in this review.

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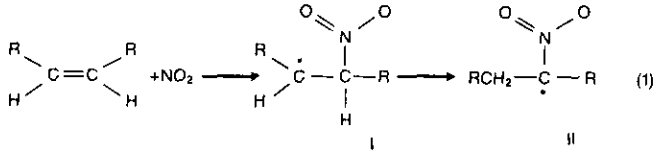
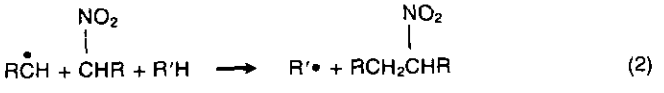
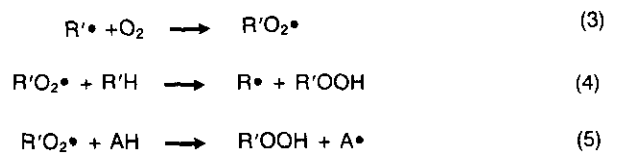
## General Aspects of Lipid Peroxidation by $NO_2$

Peroxidation of biological membrane lipids is widely considered to be an integral part of cell damage and many toxic processes (*1*). Lipid peroxidation is initiated by various free radicals and is a basic deteriorative process in living systems involving the polyunsaturated fatty acids and phospholipids in cellular membranes and other tissue structures (*2,3*). The toxicity of $NO_2$ is assumed to be related to lipid peroxidation of biomembranes, because $NO_2$ readily attacks unsaturated lipid in lung tissue. A mechanism of lipid peroxidation reaction by $NO_2$ is shown in equations 1–5 (*2*).





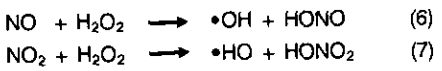
$$R'\bullet + O_2 \longrightarrow R'O_2\bullet \quad (3)$$

$$R'O_2\bullet + R'H \longrightarrow R\bullet + R'OOH \quad (4)$$

$$R'O_2\bullet + AH \longrightarrow R'OOH + A\bullet \quad (5)$$

Because the ethylene group of unsaturated fatty acids is readily attacked by $NO_2$, the free radical species shown as I and II in Equation 1 are generated. Based on spectroscopic evidence, Rowlands and co-workers (*3*) proposed that species I is converted to species II, but this type of reaction seems unlikely when compared to similar free radical reactions. Most likely, the alkyl free radicals can abstract hydrogen from unsaturated fatty acids, which leads to conventional autoxidation (*4,5*) (Eqs. 2–5). The autoxidation reaction is effectively retarded by phenolic antioxidants such as α-tocopherol, butylated hydroxyanisole, and butylated hydroxytoluene, suggesting that Equation 5 is likely.

On the other hand, both NO and $NO_2$ are known to react with hydrogen peroxide in the gas phase to produce hydroxyl radicals, as shown in Equations 6 and 7 (*6*).



$$NO + H_2O_2 \longrightarrow \bullet OH + HONO \quad (6)$$

$$NO_2 + H_2O_2 \longrightarrow \bullet HO + HONO_2 \quad (7)$$

The formation of hydroxyl radicals in the lung is feasible, because pulmonary alveolar macrophages can produce hydrogen peroxide via superoxide production in the lung when polluted air is breathed. These hydroxyl radicals are known to be extremely damaging in biological systems, as they are initiators of the lipid peroxidation reaction.

## *In Vivo* Lipid Peroxidation

Evidence that $NO_2$ causes lipid peroxidation was shown by the measurement of conjugated dienes in lungs (*7*) of rats exposed to 1 ppm $NO_2$, 4 hr daily for 6 consecutive days. The formation of lipid peroxides in the rats fed α-tocopherol-supplemented diets was lower than that of the rats fed chow diets. Since then, many investigators have tried to detect lipid peroxides in lung tissues; however, they have been unable to detect lipid peroxides following exposure to $NO_2$ (*8*).

### Acute Exposure

Recently, we confirmed the occurrence in *in vivo* lipid peroxidation following acute (*9,10*), subacute (*11*), and chronic exposure (*12,13*) to $NO_2$ by the measurement of ethane in the breath of rats and TBA reactants in lung homogenates. Furthermore, the occurrence of lipid peroxides was reported by Sevanian et al. by the measurement of fatty acid epoxides (*14*) in lung lipids and lung microsomes (*15,16*) of rats exposed to $NO_2$. We reported that time-dependent changes of lipid peroxidations as measured by ethane exhalation and thiobarbituric acid (TBA) reactants in lungs of rats exposed to 10 ppm $NO_2$ for 2 weeks varied widely during the exposure (*9,10*) (Fig. 1). The periodic changes of glutathione peroxidase activity are also depicted in Figure 1. Ethane exhalation decreased significantly on day 1. This decrease in the early phase is confirmed in another experiment in which rats were exposed to 4.48 ppm $NO_2$ for 1 hr (*17*). Thereafter, ethane exhalation increased rapidly after 2 days and reached a maximum level at 3 to 4 days. The ethane exhalation began to decrease and returned to the initial level at 10 days.

TBA reactants in lung tissue also decreased at day 1 and began to increase from 2 to 3 days. Maximum TBA was observed at 3 days, and then the value decreased rapidly and returned to the initial level. Figure 1 shows the difference in increased percentage between the TBA reactants and ethane exhalation. Figure 1 also shows the formation of lipid peroxides in organs other than lungs from 4 to 7 days, showing a maximum at 5 days, because ethane exhalation reflects the total lipid peroxidation occurring in the entire body. This time course of TBA reactants was similar to the proliferation of type II cells in alveolar tissues following 2 and 17 ppm $NO_2$ exposure, as shown by Evans et al. (*18–20*). Therefore, the result of TBA reactants may correspond to the process in which Type I cells are damaged in an early phase (0–1 day) by $NO_2$ exposure; Type II cell proliferation as a repair process begins at 1 to 3 days; and Type II cell proliferation declines after 3 days. These results suggest that formation of lipid peroxides in lungs may be related closely to the process of Type II cell proliferation.

### Subacute Exposure

Lipid peroxidation upon longer term exposure to relatively low levels of $NO_2$ is reported by Ichinose and

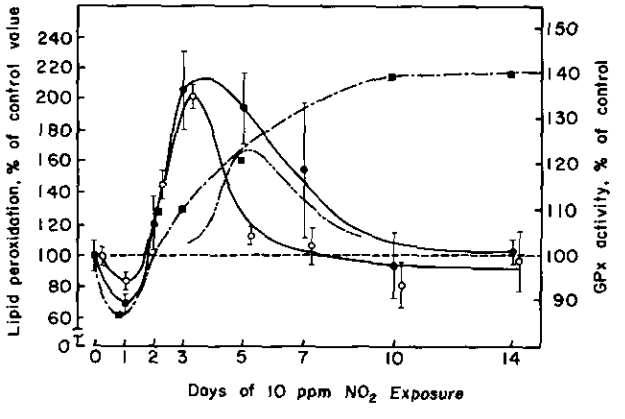



FIGURE 1. Periodic variations of ethane exhalation in breath, TBA reactants, and glutathione peroxidase activity in lung tissue of rats exposed to 10 ppm $NO_2$ for 2 weeks. Initial values were 2.07 ± 0.23 pmole/min per 100 g body weight for ethane exhalation (●) and 24.5 ± 1.5 nmole/g of lung for TBA reactants (○). Values are expressed as mean ± SE ($n$ = 6–12). (- - -) initial level; (- · - · -) difference between ethane contents and TBA reactants. (■) periodic changes of glutathione peroxidase activity.

Sagai (*11*). Wistar male rats were exposed continuously to 0.4, 1.2, and 4 ppm $NO_2$ for 1, 2, 4, 8, 12, and 16 weeks. Lipid peroxides as measured by ethane exhalation and TBA reactants in lungs are shown in Figure 2. Ethane exhalation increased to maximum levels after the first week of $NO_2$ exposure. At 4 weeks, ethane exhalation had returned to near the initial level, but tended to increase again very gradually from 8 to 16 weeks. The slight, time-dependent increases of ethane exhalation in the control group may be due to aging effect.

Maximum levels of TBA reactants in lungs were observed between 2 and 4 weeks, and then returned to the initial level at 8 weeks. Thereafter, TBA showed a tendency to increase gradually from 12 to 16 weeks. A dose dependency of ethane exhalation and TBA reactants was observed throughout the study period.

## Chronic Exposure

In an experiment of life-span exposure, Wistar male rats were continuously exposed to 0.04, 0.4, and 4 ppm $NO_2$ for 9, 18, and 27 months (*12*) at each concentration. Table 1 shows the concentration of TBA reactants in lungs of rats exposed to 0.04, 0.4, and 4 ppm $NO_2$ for 9 and 18 months. The significant increase of TBA reactants in lungs at 9 months was observed in only the 4 ppm $NO_2$ group. The amounts of TBA reactants in lungs at month 18 also increased significantly in 0.4 and 4 ppm $NO_2$ groups. Furthermore, the absolute values of TBA reactants showed a tendency to increase with aging in all groups.

Ethane evolution increased significantly and in a dose-dependent fashion upon exposure to 0.04, 0.4, and 4 ppm $NO_2$ at 9 and 18 months (Fig. 3). Ethane evolution at 27 months also increased significantly and in a dose-dependent fashion upon exposure to 0.04 and 0.4 ppm $NO_2$. However, ethane evolution in the 4 ppm $NO_2$ group was lower than in the 0.04 and 0.4 ppm $NO_2$ groups. Values for the 4 ppm group were significantly lower than the values for the 0.04 and 0.4 ppm $NO_2$ groups, but were not different from the control values at 27 months. This return to the control level did not mean recovery to the normal state of the lungs. At 27

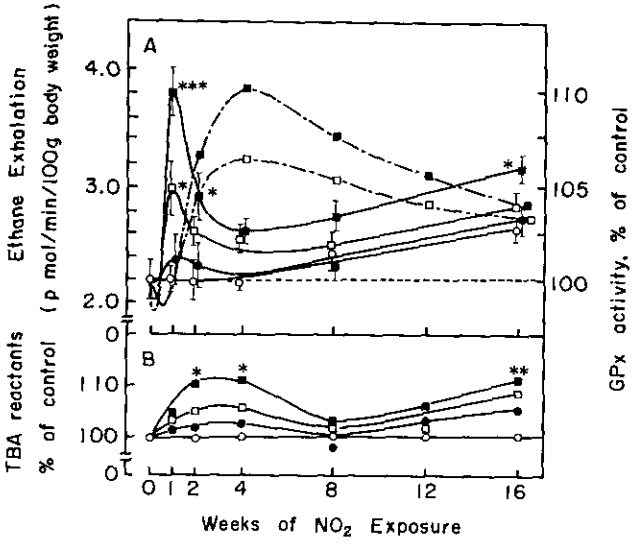



FIGURE 2. Periodic changes of ethane exhalation in breath, glutathione peroxidase activity (*A*), and TBA reactants (*B*) in lungs of rats exposed to 0 (control), 0.4, 1.2, and 4 ppm $NO_2$ for 4 months. (—) in *A* ethane exhalation; (—) in *B* shows TBA reactants; (- - - -) glutathione peroxidase activity. (○) control group; (●) 0.4 ppm group; (□) 1.2 ppm group; (■) 4 ppm group; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

**Table 1. Concentration of thiobarbituric acid (TBA) reactants in rat lungs after chronic exposure to nitrogen dioxide.**

| Exposure group | TBA values nmole/g of wet lung | | | |
|---|---|---|---|---|
| | 9-month exposure | | 18-month exposure | |
| Control | 46.2 ± 2.3[a] | (100%) | 50.9 ± 1.6 | (100%) |
| 0.04 ppm | 45.3 ± 2.2 | (98%) | 54.3 ± 1.3 | (107%) |
| 0.4 ppm | 46.8 ± 2.5 | (101%) | 59.6 ± 2.0** | (117%) |
| 4.0 ppm | 53.8 ± 3.8* | (116%) | 63.8 ± 2.5** | (125%) |

[a] $n$ = 12 rats per group. Values are $\bar{x}$ ± SE. Numbers in parentheses are percent of control value.

*$p < 0.05$, statistical significance between $NO_2$-exposed group and control group was determined by Student's $t$-test.

**$p < 0.01$.

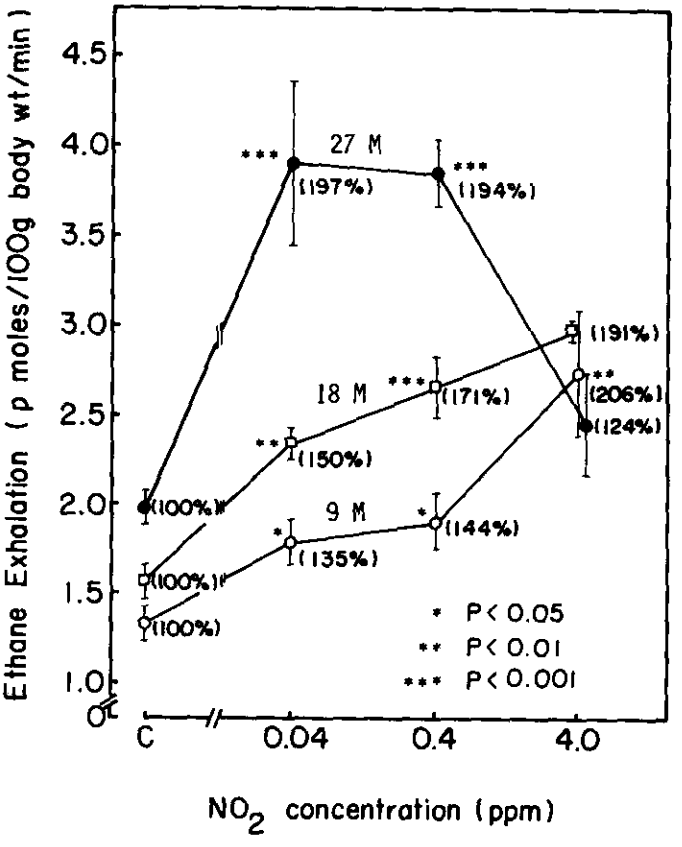


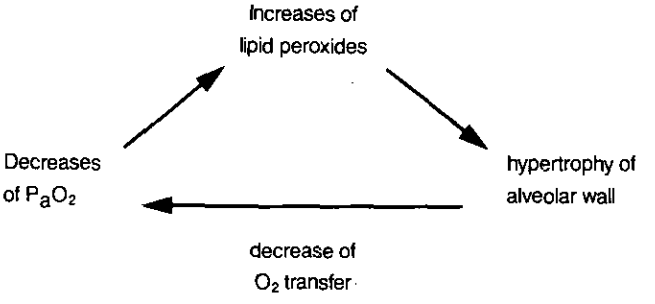

FIGURE 3. Ethane exhalation in expired gases of rats exposed continuously to 0.04, 0.4, and 4 ppm $NO_2$ for 9, 18, and 27 months. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

months the decrease of the mean thickness of the alveolar wall and the appearance of lung fibrosis were observed by Takenaka et al. (*21*) (Fig. 4) in the pathological examination of this study program. Therefore, the decrease of ethane exhalation of rats exposed to 4 ppm $NO_2$ for 27 months may be partially due to the decrease of ventilatory capacity by lung fibrosis. However, such marked decrease of ethane exhalation might not be explained by only the decrease of ventilatory capacity. The decrease of lipid peroxidation itself may also be a cause of the marked decrease of ethane exhalation. From these results, it was shown that lipid peroxidation, as measured by ethane exhalation in the breath of rats, is an excellent index of a biochemical effect of the exposure to lower levels of $NO_2$. It was shown that the increments of ethane exhalation were clearly related to the rise of $NO_2$ concentrations and prolongation of the exposure periods within an 18-month exposure (Fig. 3). Furthermore, these changes were similar to that pattern of the increased arithmetic mean thickness of the alveolar wall observed by Takenaka et al. (*21*). Hypertrophy of alveolar wall may relate to the formation of lipid peroxides in alveolar wall. Bils (*22*) also reported a thickening of the collagen fibrils in squirrel monkeys exposed to 3 ppm $NO_2$, 4 hr daily for 4 days.

Although there is no direct relevancy to $NO_2$, the relation between lipid peroxidation and hypertrophy in retina of chick embryo were reported by Yagi et al. (*23*). They exposed chick embryos to a high concentration of oxygen to examine the causes of retrolental fibroplasia. These researchers observed the hypertrophy of the retina and the increment of lipid peroxides in retina. This result also indirectly suggests that the hypertrophy of alveolar wall may relate to the increment of lipid peroxides.

Suzuki et al. (*24,25*) observed that arterial blood oxygen tension ($Pa_{O_2}$) of rats exposed to 0.4 and 4.0 ppm $NO_2$ for 9 and 18 months, respectively, was decreased significantly from the control, but arterial carbon dioxide tension ($Pa_{CO_2}$) and $pH_a$ did not change, as shown in Figure 5. Decreases of $Pa_{O_2}$ in rats, rabbits, and humans exposed to $NO_2$ were also reported by Freeman et al. (*26*), Davidson et al. (*27*), and Nieding and Wagner (*28*), respectively. The decrease of $Pa_{O_2}$ may be induced by the thickening of the alveolar wall. Yoshikawa et al. (*29,30*) reported that TBA reactants in serum, abdominal aorta, and brain of rats were increased significantly with a decrease of $Pa_{O_2}$ under conditions of low oxygen supply. These data indirectly suggest that rats exposed to 0.4 and 4 ppm $NO_2$ for 9 and 18 months exhibit a hypoxemia-like condition and that lipid peroxidation may be stimulated by such chronic hypoxemia.

Overall, these results suggest that chronic lung diseases such as lung fibrosis may progress by a positive feedback of chronic effects such as the increases of lipid peroxides, hypertrophy of alveolar wall, and the decreases of $Pa_{O_2}$, as shown below.



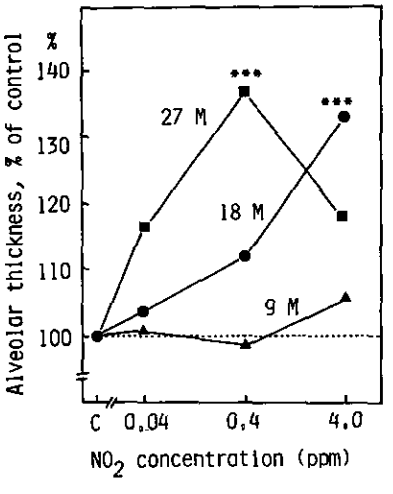




FIGURE 4. Arithmetic mean thickness of alveolar wall of rats continuously exposed to 0.04, 0.4, and 4 ppm $NO_2$ for 9, 18, and 27 months.

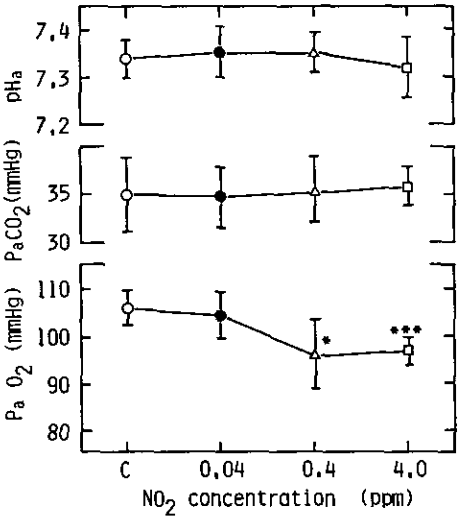
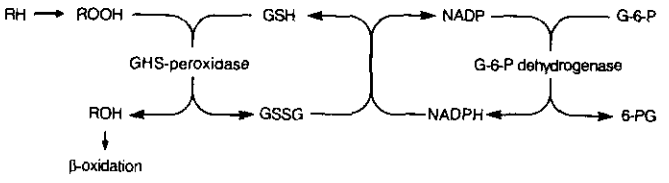



FIGURE 5. Effect of $NO_2$ exposure on arterial blood $pH_a$, $Pa_{CO_2}$, and $Pa_{O_2}$ in unanesthetized rats. Values are expressed as mean ± SD ($n = 6$); (C) control; $*p < 0.05$; $***\ p < 0.001$.

## Effects of Enzyme Systems as Biochemical Protective Mechanisms

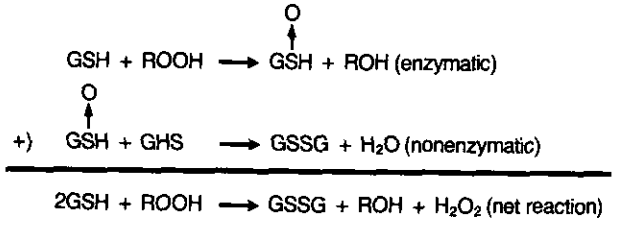
The role of enzyme systems that can metabolize lipid peroxides or inhibit their formation is very important for protecting cells from oxidative stress. The glutathione peroxidase system, consisting of glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase, is a typical enzyme system protecting against oxidative damage. Chow and Tappel (*31*) proposed an enzymatic system for the protection of the lung against lipid peroxidation damage by ozone.

Glutathione peroxidase is considered to be responsible for the detoxification of lipid peroxides in tissue, thus protecting cellular components from oxidative damage (*32–34*).




Net Reaction: ROOH + 2GSH → ROH + GSSG + $H_2O$
Where RH shows polyunsaturated fatty acid,
and ROOH shows lipid hydroperoxides

Glutathione *S*-transferase also protects cells from oxidative stress, because it can catalyze the same net reaction as glutathione peroxidase (*35,36*).




Superoxide dismutase provides the basic defense against the potential cytotoxic reactivities of superoxide anion radicals (*37*). An increase of superoxide dismutase activity in lungs may represent adaptive changes that reduce oxidative damage. DeLucia et al. (*38*) reported that mixed disulfide between protein sulfhydryls and nonprotein sulfhydryls was formed by ozone exposure. The mixed disulfide formed is reduced to each free sulfhydryl by disulfide reductase (*39*); therefore, disulfide reductase also plays an important role in the reduction of the mixed disulfide produced by oxidative stress (*38,39*).

The effects of $NO_2$ on these enzymatic protective systems were first reported by Chow et al. (*40*), Menzel et al. (*41*), and Fukase et al. (*42*). Their results revealed that glutathione peroxidase can be induced only by exposure to relatively high levels of $NO_2$. Furthermore, Chow et al. (*40*) suggested that $NO_2$ mainly attacks reducing substances such as glutathione and NADPH. Recently, we reported the alteration of these protective enzymes in lungs of rats exposed to a relatively high level (10 ppm) of $NO_2$ for 2 weeks. The time-dependent changes of the activities of glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase in lungs of rats exposed to 10 ppm $NO_2$, as shown in Figure 6, were nearly symmetric with ethane exhalation and TBA reactants. The symmetric relationship suggests that lipid peroxides produced by the exposure to $NO_2$ induce these enzyme activities to protect cells from oxidative damage (*10*). Although McCay et al. (*43*) reported that glutathione peroxidase would not reduce lipid hydroperoxides present in membranes, Tappel (*44*) explored this phenomenon and found new evidence for a phospholipase that hydrolyzed fatty acid hydroperoxides from phospholipids at rates significantly faster than those of known phospholipases. The existence of a phospholipase with a faster hydrolysis rate may explain this discrepancy.

Sevanian et al. (*45*) reported that lipid hydroperoxides originating in the membrane were effectively reduced by glutathione peroxidase when phospholipase $A_2$ was present in the assay system, and that low level glutathione peroxidase activity was observed in the absence of phopholipase $A_2$. These findings might explain a suitability of the symmetrical changes between ethane formation and glutathione peroxidase activity. The increased activities of the enzymes illustrated in Figure

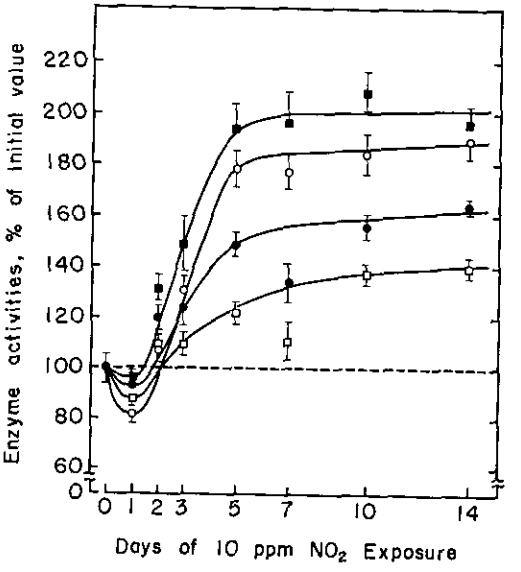



FIGURE 6. Periodic variations of glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase activities. Initial values (nmole of $NADP^+$ reduced per mg of protein per min) were 88.8 ± 2.9 for GPx(□); 124.3 ± 3.5 for GR(●); 126.6 ± 5.9 for G6PD(■); and 145.4 ± 5.6 for 6PGD (○). Each value is expressed as mean ± SE (N = 6–12).

6 may be a compensatory reaction for protecting cells from lipid peroxide-induced damages.

The time-dependent changes of superoxide dismutase and disulfide reductase in lungs of rats exposed to 10 ppm $NO_2$ are similar to those of glutathione peroxidase and glutathione reductase. The induced activity of superoxide dismutase indirectly suggests involvement of the superoxide anion radical in the formation of lipid peroxidation products and the deterioration of cells after $NO_2$ exposure. However, the involvement might be small because the induction of superoxide dismutase was slight. The induction of disulfide reductase was remarkable; therefore, the formation of mixed disulfide by $NO_2$ exposure might be largely due to the exposure to relatively high levels of $NO_2$.

Changes in glutathione peroxidase, glutathione reductase (Fig. 7*A*), glucose-6-phosphate dehydrogenase (Fig. 7*B*), superoxide dismutase, and disulfide reductase in lungs of rats exposed continuously to 0.4, 1.2, and 4 ppm $NO_2$ for 16 weeks were examined. The maximum levels of these protective enzyme activities were observed at the 4th week, and then the activities of the antioxidative protective enzymes showed a tendency to decrease gradually with prolongation of exposure period. The temporal changes between the antioxidative protective enzyme activities and lipid peroxidation varied inversely. Such inverse changes have also been observed in the acute exposure of $NO_2$, as shown in Figures 1 and 6. These results also suggest that the induction of the antioxidative protective enzymes is a compensatory reaction against lipid peroxide-induced damage.

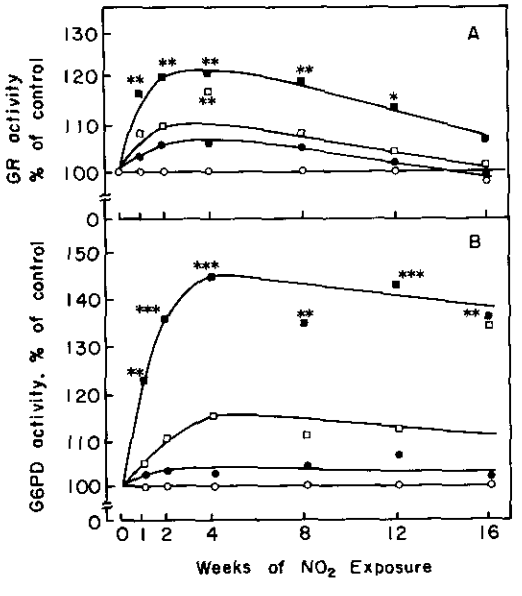



FIGURE 7. Time-dependent changes in glutathione reductase (GR) and glucose-6-phosphate dehydrogenase activities in lungs of rats exposed to 0, 0.4, 1.2, and 4.0 ppm $NO_2$ for 4 months. Control values of GR activities were between 110 and 78 nmole of $NADP^+$ formed/mg protein/min, and control values of G6PD activities were between 69.5 and 44.8 nmole of NADPH formed/mg protein/min, from 1 through 16 weeks. (○) control group; (●) 0.4 ppm group; (□) 1.2 ppm group; (■) 4.0 ppm group. $*p < 0.05$; $**p < 0.01$.

We also examined the changes of the antioxidative protective enzyme activities in lungs of rats exposed to 0.04, 0.4, and 4 ppm $NO_2$ for a life-span (*12*). The results obtained are shown in Tables 2 and 3. Glutathione peroxidase activity measured by using cumene-hydroperoxide as a substrate ($GP_x$-cumene·OOH) did not show any significant changes at months 9 and 18. The activity of glutathione peroxidase measured by using hydrogen peroxide as a substrate ($GP_x$-$H_2O_2$) decreased below each control level in lungs of rats exposed to 4 ppm $NO_2$ for 9 months and 0.4 and 4 ppm $NO_2$ for 18 months. The activities of glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PD), and 6-phosphogluconate dehydrogenase (6PGD) in the 9-month exposure group generally showed increases with an elevation of $NO_2$ level, and significant increases in glutathione reductase and glucose-6-phosphate dehydrogenase were observed in the group exposed to 4 ppm $NO_2$. A significant increase of glucose-6-phosphate dehydrogenase was also observed in the 4 ppm $NO_2$ group after the 18-month exposure. However, values for these three enzymes in the other groups returned to their control level at the 18-month exposure. The activities of three kinds of glutathione *S*-transferases at 9 months did not show any significant changes among the three groups of $NO_2$ exposure, but the enzyme activities at 18 months, except epoxy *S*-transferase, decreased significantly below the control level in 0.4 and 4 ppm $NO_2$ groups (Table 3).

It is reported that the activities of superoxide dismutase and disulfide reductase did not show any significant changes. Ayaz and Csallany (*46*) exposed female mice continuously for 17 months to 0.5 and 1 ppm $NO_2$ and fed the animals a basal diet which was either deficient in vitamin E or supplemented with 30 and 300 mg/kg of diet. Consistent with our results, they found the suppression of glutathione peroxidase in mice lung of a combined group of vitamin E deficiency and 1 ppm $NO_2$ exposure. These results show that the activities of the antioxidative protective enzymes, especially glutathione peroxidase and glutathione *S*-transferase, tended to decrease with prolongation of exposure period, and that lipid peroxidation conversely increased with prolongation of exposure period. From these results, we proposed the overall relationship between the antioxidative protective enzymes and lipid peroxidation (*12*), as shown in Figure 8.

## Effects on Antioxidants

Reducing substances, such as NADPH, NADH, glutathione, and vitamin C (ascorbic acid) are important for the maintenance of reducing potential and protection of cells against oxidative stress.

Increase of reducing substances such as NADPH and glutathione in lung tissue of the animals exposed to $NO_2$ can be predicted easily from the increased activities of glucose-6-phosphate dehydrogenase and glutathione reductase, as described previously. Ospital et al. (*47*) re-

**Table 2. Concentration of total protein and the activities of glutathione peroxidase (GPx), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PD), and 6-phosphogluconate dehydrogenase (6-PGD) in rat lungs after chronic exposure to nitrogen dioxide.**

| Parameter | Exposure group | 9-month exposure | | 18-month exposure | |
|---|---|---|---|---|---|
| Total protein | Control | 89.2 ± 2.1[a] | (100%) | 89.1 ± 2.8 | (100%) |
| mg/g wet lung | 0.04 ppm | 87.2 ± 1.8 | (98%) | 91.7 ± 2.5 | (100%) |
| | 0.4 ppm | 92.7 ± 3.3 | (104%) | 96.1 ± 1.9 | (105%) |
| | 4.0 ppm | 94.3 ± 2.5 | (106%) | 89.0 ± 1.7 | (100%) |
| GPx-cumene·OOH[b] | Control | 12.56 ± 0.17 | (100%) | 12.48 ± 0.94 | (100%) |
| μmole NADPH | 0.04 ppm | 12.97 ± 0.38 | (103%) | 13.36 ± 1.05 | (107%) |
| oxidized/min/g | 0.4 ppm | 12.63 ± 0.44 | (101%) | 12.17 ± 0.80 | (98%) |
| wet wt. of lungs | 4.0 ppm | 13.13 ± 0.36 | (105%) | 11.72 ± 0.70 | (94%) |
| GPx-$H_2O_2$[c] | Control | 8.12 ± 0.17 | (100%) | 9.05 ± 0.3 | (100%) |
| μmole NADPH | 0.04 ppm | 7.61 ± 0.21 | (94%) | 9.59 ± 0.37 | (106%) |
| oxidized/min/g | 0.4 ppm | 7.45 ± 0.37 | (92%) | 7.21 ± 0.15** | (80%) |
| wet wt. of lungs | 4.0 ppm | 7.19 ± 0.27* | (89%) | 8.10 ± 0.20* | (89%) |
| GR | Control | 5.24 ± 0.07 | (100%) | 5.50 ± 0.12 | (101%) |
| μmole NADPH | 0.04 ppm | 5.25 ± 0.06 | (100%) | 5.57 ± 0.18 | (101%) |
| oxidized/min/g | 0.4 ppm | 5.47 ± 0.13 | (104%) | 5.04 ± 0.14 | (101%) |
| wet wt. of lungs | 4.0 ppm | 6.00 ± 0.11*** | (115%) | 6.00 ± 0.17 | (109%) |
| G6PD | Control | 3.18 ± 0.16 | (100%) | 4.40 ± 0.25 | (100%) |
| μmole NADPH | 0.04 ppm | 3.21 ± 0.12 | (101%) | 5.02 ± 0.20 | (114%) |
| formed/min/g wet | 0.4 ppm | 3.53 ± 0.15 | (111%) | 4.80 ± 0.18 | (111%) |
| wt. of lungs | 4.0 ppm | 4.72 ± 0.18*** | (148%) | 5.66 ± 0.33** | (128%) |
| 6 PGD | Control | 3.94 ± 0.17 | (100%) | 4.67 ± 0.13 | (100%) |
| μmole NADPH | 0.04 ppm | 4.05 ± 0.11 | (103%) | 4.85 ± 0.12 | (104%) |
| formed/min/g wet | 0.4 ppm | 3.78 ± 0.21 | (96%) | 4.68 ± 0.17 | (100%) |
| wt. of lungs | 4.0 ppm | 4.41 ± 0.13 | (114%) | 5.15 ± 0.16 | (110%) |

[a] $n = 12$ rats per group. Values are $\bar{x} \pm$ SE. Numbers in parentheses are percent of control value.
[b] GPx-cumene·OOH shows glutathione peroxidase assayed by cumene hydroperoxide as substrate.
[c] GPx-$H_2O_2$ shows glutathione peroxidase assayed by hydrogen peroxide as substrate.
*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$.

**Table 3. Activities of three glutathione S-transferases: aryl, aralkyl, and epoxy S-transferase, in rat lungs after chronic exposure to nitrogen dioxide.**

| Type of transferase | Exposure group | 9-month exposure | | 18-month exposure | |
|---|---|---|---|---|---|
| Aryl S-transferase | Control | 0.179 ± 0.004[a] | (100%) | 0.199 ± 0.008 | (100%) |
| μmole/min/g wet | 0.04 ppm | 0.187 ± 0.005 | (101%) | 0.194 ± 0.009 | (98%) |
| wt. of lung | 0.4 ppm | 0.187 ± 0.005 | (104%) | 0.170 ± 0.008* | (89%) |
| | 4.0 ppm | 0.172 ± 0.005 | (96%) | 0.153 ± 0.009** | (77%) |
| Aralkyl S-transferase | Control | 2.53 ± 0.07 | (100%) | 2.39 ± 0.10 | (100%) |
| μmole/min/g wet | 0.04 ppm | 2.54 ± 0.05 | (100%) | 2.32 ± 0.12 | (97%) |
| wt. of lung | 0.4 ppm | 2.65 ± 0.06 | (105%) | 2.07 ± 0.11* | (86%) |
| | 4.0 ppm | 2.50 ± 0.09 | (99%) | 2.00 ± 0.07** | (84%) |
| Epoxy S-transferase | Control | 0.143 ± 0.010 | (100%) | 0.170 ± 0.010 | (100%) |
| μmole/min/g wet | 0.04 ppm | 0.154 ± 0.007 | (108%) | 0.340 ± 0.030 | (117%) |
| wt. of lung | 0.4 ppm | 0.161 ± 0.012 | (113%) | 0.190 ± 0.020 | (112%) |
| | 4.0 ppm | 0.133 ± 0.006 | (93%) | 0.186 ± 0.010 | (109%) |

[a] $n = 12$ rats per group. Values are $\bar{x} \pm$ SE. Numbers in parentheses are percent of control value.
* $p < 0.05$.
**$p < 0.01$.

ported increase of the activity of the glycolytic pathway in lung slices of rats exposed to 5 ppm $NO_2$. They suggested that this increased activity may be due to an increased enzyme biosynthesis to protect cells from injury upon $NO_2$ exposure. It is well known that various kinds of sulfur-containing compounds also reduce the toxicity of $NO_2$ (*48,49*).

With regard to the interaction between glutathione and ascorbic acid, Leung and Morrow (*50*) showed that dehydroascorbic acid can be reduced to ascorbic acid by glutathione *in vitro*, but oxidized glutathione cannot be reduced to glutathione by ascorbic acid. Vitamin E, as well as vitamin C, is important as an antioxidant that reacts rapidly with organic free radicals (*51–54*). Vitamin C levels in the tissue are often considerably greater than those of vitamin E (*55*). Nevertheless, vi-

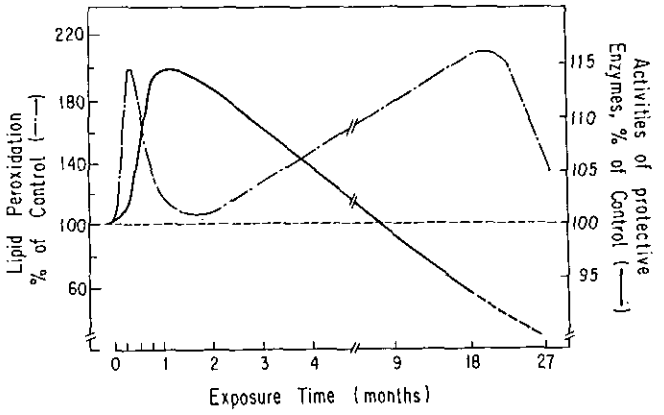



FIGURE 8. The temporal changes of lipid peroxidation as measured by ethane exhalation and activities of antioxidative protective enzymes of rats exposed to 4 ppm $NO_2$ for their life-span. (—·—·—) lipid peroxidation; (——) activities of the antioxidative protective enzymes.

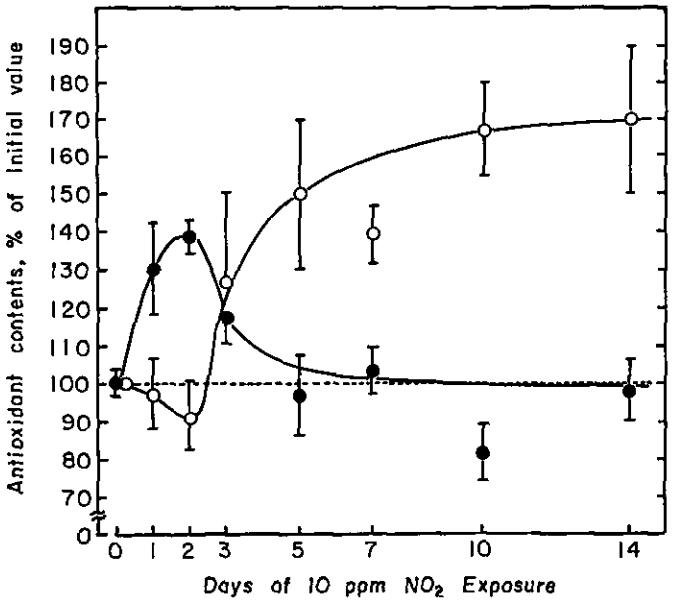



FIGURE 9. Periodic variations of nonprotein sulfhydryls and vitamin E in the lungs. Initial values of nonprotein sulfhydryls (○) and vitamin E (●) were 11.1 ± 0.05 μmole/g lung and 33.2 ± 1.1 μg/g lung, respectively. Values are expressed as mean ± SE ($n$ = 6–12).

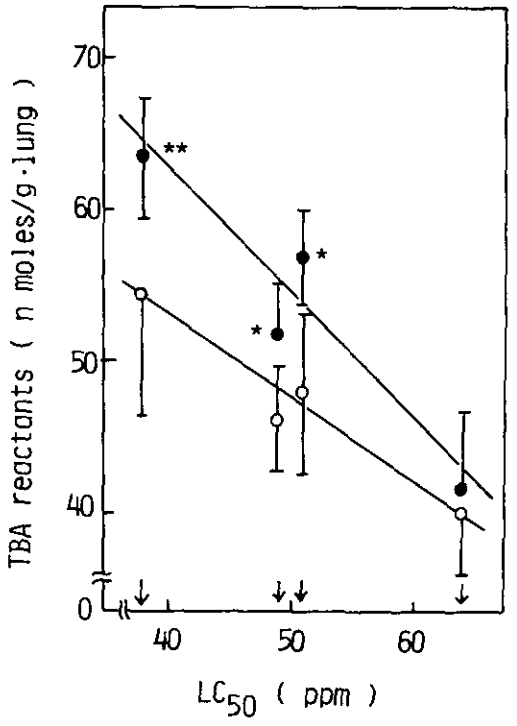
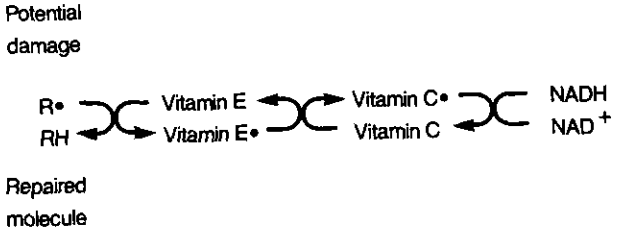



FIGURE 10. The relationship between the concentration of TBA reactants in the lungs of the four strains of mice and their $LC_{50}$. $LC_{50}$ values have been reported previously (*67*) to be 38, 49, 51, and 64 ppm for ICR, BALB/c, ddy, and C57BL/6, respectively. Values for the control group (○) and exposed (●) group are expressed as mean ± SD ($n$ = 6). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

tamin E, which is considerably more lipophilic, has been found to be the more potent antioxidant in membranes.

Menzel (*56,57*) proposed that vitamin E as an antioxidant might protect the lung from damage by $NO_2$ by inhibiting lipid peroxidation. Data related to this hypothesis have been reported by many investigators (*7,46,56–60*). Tappel (*60*) has suggested synergistic action of the two vitamins with regard to the action of vitamin E as the primary antioxidant and the regeneration of vitamin E radical by the reaction with vitamin C. Recently, Packer et al. (*61*) proposed the following scheme from the result of vitamin C oxidation by electron transportation to phenoxy radicals of vitamin E, as shown below.




Vitamin C plays an important role in the maintenance of vitamin E levels in tissue. It is believed that vitamin E and vitamin C protect cells synergistically or cooperatively against oxidative stress.

The amounts of nonprotein sulfhydryls (mainly reduced glutathione) and vitamin E as antioxidants in lungs of rats exposed to 10 ppm $NO_2$ for 2 weeks were reported (*10*) (Fig. 9). The time course of the nonprotein sulfhydryls was very similar to that of protective enzymes. In contrast, the time course of vitamin E was similar to that of lipid peroxidation in Figure 1, and it was symmetric to that of nonprotein sulfhydryls.

This result suggests that vitamin E is an important

**Table 4. $LC_{50}$ in various strains of animals for $NO_2$ exposure for 16 hr.**

| Animal | Strain | Sex | $LC_{50}$, ppm |
|---|---|---|---|
| Mouse | C57BL/6 | F | 67 |
| | C57BL/6 | M | 64 |
| | BDF1 | F | 60 |
| | CDF1 | F | 59 |
| | C3H/He | M | 57 |
| | BDF1 | M | 56 |
| | CDF1 | M | 56 |
| | BALB/c | F | 52 |
| | DBA/2 | M | 52 |
| | ddy | M | 51 |
| | C3H/He | F | 50 |
| | BALB/c | M | 49 |
| | ddy | F | 48 |
| | DBA/2 | F | 45 |
| | ICR | F | 40 |
| | ICR | M | 38 |
| | CF#1 | M | 36 |
| | CF#1 | F | 33 |
| Hamster | Golden | M | 28 |
| | Golden | F | 22 |
| Rats | Fischer | M | 56 |
| | SD | M | 50 |
| | Wistar | M | 49 |
| | Fischer | F | 48 |
| | SD | F | 47 |
| | Donryu | M | 47 |
| | Wistar | F | 45 |
| | Donryu | F | 39 |
| Guinea pig | Hartley | M | 62 |
| | Hartley | F | 50 |

factor that acts at an early stage to prevent the formation of lipid peroxides. The increased amount of vitamin E might be transported from other organs such as liver, because vitamin E cannot be synthesized by the animal's body. The authors guess that both nonprotein sulfhydryls and vitamin E act mutually as complementary factors to protect cells from oxidative damage. The time-dependent changes of nonprotein sulfhydryls and vitamin E in the subacute experiment were similar to that of glutathione-related enzyme activities shown in Figure 7, and lipid peroxidation as measured by ethane exhalation shown in Figure 2, respectively.

In the life-span exposure of 0.04, 0.4, and 4 ppm $NO_2$, the significant increase of nonprotein sulfhydryls was observed at 4 ppm $NO_2$ at the 9 and 18 months (*12*). In contrast, Nakajima et al. (*62*) have reported that the amounts of reduced glutathione in lungs of mice continuously exposed to 0.7 to 0.8 ppm $NO_2$ over 6 months decreased below the control level at the stage of body weight loss. These results suggest that there is a species difference in the protective ability of reduced glutathione against the toxicity of $NO_2$. We found that the protective ability of reduced glutathione against $NO_2$ was different not only among various species (*63*), but also strains of animals (*64*). [See also references (*65,66*) on species differences of the contents of lipid peroxides, antioxidants, and phospholipids of various control animals.]

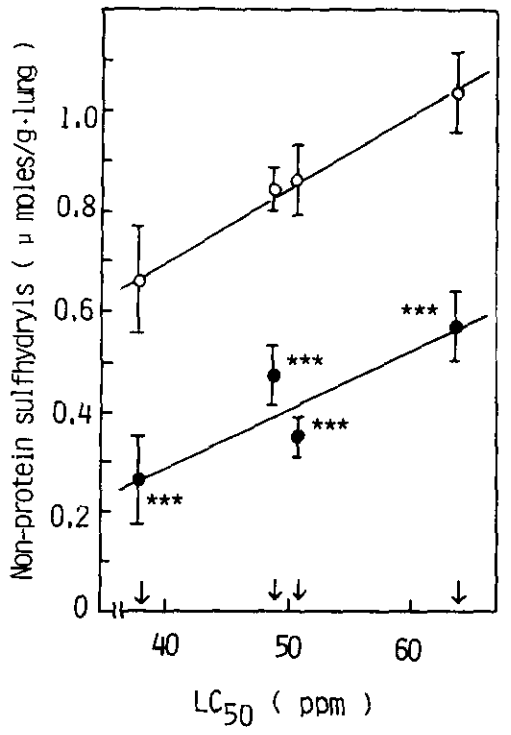



FIGURE 11. The relationship between the concentration of NPSH in lungs of the four strains of mice and their $LC_{50}$. $LC_{50}$ values have been reported previously (*67*) to be 38, 49, 51, and 64 ppm for ICR, BALB/c, ddy, and C57BL/6, respectively. Values in the control group (●) are expressed as mean ± SD ($n$ = 6). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

$LC_{50}$ values of various animals species obtained by $NO_2$ exposure for 16 hr are reported by Takenaka et al. (*67*), as shown in Table 4. Furthermore, we reported that the changes of the antioxidative protective enzymes, lipid peroxides (Fig. 10), nonprotein sulfhydryls (Fig. 11), vitamin E, and total lipids in lungs of ICR, BALB/c, ddy and C57BL/6 mice exposed to 20 ppm $NO_2$ for 16 hr were closely related to the susceptibility against $NO_2$ at $LC_{50}$ (*67*).

With regard to the effect of $NO_2$ on glutathione and ascorbic acid, Leung and Morrow (*47*) reported that vitamin C in lungs of guinea pigs exposed to 45 ppm $NO_2$ for 3 hr decreased markedly, but glutathione in the lungs did not. Selgrade et al. (*68*) reported that vitamin C-deficient guinea pigs exposed to 1, 3, and 5 ppm $NO_2$ for 72 hr caused marked increase in lavage proteins and lipids, but not at the 0.4 ppm level. Fifty percent of the vitamin C-deficient animals exposed to 5 ppm died, and these animals had proteinaceous edema fluid in the alveoli. These results confirm that vitamin C also plays an important role on the protection against $NO_2$.

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