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INVOLVEMENT OF GLUTATHIONE ENZYMES IN O_2 TOLERANCE DEVELOPMENT BY DIETHYLDITHIOCARBAMATE*

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Abstract—Diethyldithiocarbamate (DDC) in high doses (≥ 250 mg/kg) accelerated the onset of death in adult rats exposed to 95% O_2. At lower doses (100 and 200 mg/kg) no increase in O_2 toxicity was seen. We observed that, at a level of 250 mg/kg, DDC accelerated the death of 26 per cent of the animals but a large number (50 per cent) developed resistance to the lethal effects of 95% O_2. These animals also demonstrated an acceleration of the rate of increase in activity of glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase activity in response to O_2, compared to untreated controls. No acceleration in the rate of increase of superoxide dismutase activity was seen. These data support a role of the glutathione-related enzymes in promoting O_2 tolerance in rats.

Exposure of adult rats to hyperoxic environments has been shown to stimulate various protective enzymes including superoxide dismutase (SOD), glutathione peroxidase (GSH peroxidase), glutathione reductase (GSSG reductase) and glucose-6-phosphate dehydrogenase (G6PD) [1–4]. At sublethal levels of 85% O_2, these enzymes in rats are significantly stimulated at 5 days of exposure, and the animals have developed a tolerance to higher O_2 levels (95–100%) that would normally be lethal. The increase in the above enzymes has been suggested by various investigators to be responsible for the induced O_2 tolerance [1, 4, 5]. Various other treatments can also increase resistance to the lethal effects of O_2, including pre-exposure to hypoxia or oleic acid [5–7], or endotoxin, administered either prior to or concomitant with the start of O_2 exposure [8–10]. In general, one or more of the above enzymes has been shown to be induced when O_2 tolerance is developed by these methods [5].

We have examined the effect of the sulfhydryl compound diethyldithiocarbamate (DDC) on the response of adult rats to hyperoxia. DDC is the reduced monomer of disulfiram (DSF) (tetraethylthiuram disulfide), popularly known as Antabuse and widely used in alcohol aversion therapy. Disulfiram is rapidly metabolized to DDC *in vivo* [11]. DDC is a strong chelator of copper and can inhibit various copper-containing enzymes *in vitro* [12] or, at high dose levels, *in vivo* [12, 13]. Frank *et al.* [13] have reported inhibition of SOD and cytochrome oxidase in neonatal or adult rat lungs by DDC at levels of 250 mg/kg body wt and higher. They have also reported increased O_2 toxicity in young rats at these levels of DDC.

We have examined further the relationship of DDC and O_2 toxicity in adult rats and have found that DDC at 200 mg/kg body wt or less has no apparent effect on the toxic response to oxygen. There is a marked acceleration of the lethal effect of O_2 with 250 mg DDC/kg body wt or more, but with 250 mg/kg body wt, treatment with DDC increases the percentage of long-term O_2 survivors. We have also found that, at the 250 mg/kg body wt dose, DDC + O_2 accelerates the rate of increase in activity of enzymes involved in maintaining the ratio of reduced to oxidized glutathione, thus providing a possible mechanism for development of tolerance in these animals.

MATERIALS AND METHODS

Animals. Charles River Sprague–Dawley-derived specific-pathogen-free male rats weighing 200–250 g at the beginning of each experiment were used. The rats were injected with DDC (Sigma Chemical Co., St. Louis, MO) that was dissolved in 0.5 to 2.0 cm^3 of sterile distilled water, immediately prior to O_2 exposure. Control animals were either untreated or injected with an equivalent volume of sterile 0.15 M saline. No differences were observed between these two control groups.

The rats were exposed to O_2 in enclosed plexiglas chambers of 30 × 30 × 45 cm with a maximum of eight rats per chamber. Humidified oxygen was allowed to flow through the chamber at a rate (8–12 l/min) sufficient to maintain the concentration at 95–97% oxygen, and soda lime was added to the bottom of the cages to maintain the CO_2 levels below 1%. O_2 and CO_2 concentrations were monitored periodically. Animals exposed to air were treated in a separate chamber with air flow instead of oxygen flow. Similar numbers of DDC-treated and control animals were placed in both the O_2 and air flow chambers. The exposure to O_2 or air was continuous except for periodic opening of the chambers for 5–10 min to remove dead animals or change the bedding.

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Tissue preparation. For enzyme analysis, live animals were removed from the chambers at given intervals of time, anesthetized with sodium pentobarbital, and killed by opening the pleural cavity. The lungs were perfused *in situ* through the pulmonary artery with physiological saline to remove blood from the tissue. The lungs were then removed from each animal, trimmed of larger airways and connective tissue, and homogenized for 2 min on ice in a Sorvall Omnimixer at setting 6 in approximately 40 ml of medium containing 5 mM Tris at pH 7.5, 0.15 M sucrose, 0.15 M mannitol and 1 mM EDTA [1]. The homogenate was centrifuged at 500 *g* and the pellet discarded. The supernatant fraction was then centrifuged at 25,000 *g* and the final supernatant fraction was used for enzyme assays.

Enzyme assays. SOD was assayed by measuring the inhibition of cytochrome *c* reduction caused by superoxide ions generated by purine oxidation by xanthine oxidase. Conditions for this assay were as described by McCord and Fridovich [14] and modified by the use of 10^{-5} M KCN to inhibit cytochrome oxidase [15]. One unit of enzyme activity is defined as 50 per cent inhibition of the standard rate of reduction of cytochrome *c* as established by McCord and Fridovich [14]. Alternatively, the SOD levels were measured by the epinephrine method described by Misra and Fridovich [16] which measures primarily the cupro-zinc form of the SOD. One unit of SOD causes 50 per cent inhibition of the auto-oxidation of epinephrine as defined by Misra and Fridovich [16].

GSH peroxidase activity in the supernatant fraction was measured as described by Little *et al.* [17] by coupling the reaction of glutathione peroxidase and cumene hydroperoxide (obtained from Matheson, Coleman & Bell, Norwood, OH) with glutathione reduction and NADPH oxidation by GSSG reductase. One unit of enzyme activity is the equivalent of 1 μmole of NADPH oxidized/min at pH 7, as measured by absorbance at 340 nm on a Gilford spectrophotometer. The rate was corrected for blanks containing either no tissue or no cumene hydroperoxide. GSSG reductase was measured by a modification of the method of Horn [18] by following NADPH disappearance at pH 7.

Glucose-6-phosphate dehydrogenase (G6PD) activity was determined by following the reduction of $NADP^+$ during the oxidation of glucose-6-phosphate by the enzyme at 30° in a Gilford spectrophotometer [19], a modification of the method of Lohr and Waller [20].

Glutathione peroxidase activities were measured on freshly prepared homogenates since freezing resulted in a loss of activity. G6PD, GSSG reductase and SOD were measured either on fresh or frozen homogenates. There was no loss of activity of these enzymes in samples frozen for as long as 2 months.

All enzymes and substrates were from the Sigma Chemical Co. unless otherwise specified. All enzyme activities are expressed as total units per pair of rat lungs.

RESULTS

Table 1 shows the results of various doses of DDC on the response of rats to 95–97% O_2. For untreated control animals there were no deaths before 48 hr. The largest number of animals died between 48 and 96 hr. A small number of the untreated rats were resistant to O_2 and survived indefinitely with substantial lung damage. We found that any animal surviving longer than 96 hr in O_2 did not die for at least 2 weeks. No animals were kept longer than 3 weeks and most were killed after 6–7 days and designated 'survivors'.

The effect of O_2 on rats injected with 100 or 200 mg DDC/kg body wt prior to O_2 exposure was similar to that seen in control animals. Doses of 300 and 500 mg DDC/kg body wt accelerated deaths resulting from 95–97% O_2. At these respective doses of DDC, 59 and 84 per cent of the rats died before 24 hr of O_2 exposure. The actual time of death for these animals occurred in a narrow interval between 16 and 23 hr of O_2 exposure. Animals that received the higher doses of DDC and were still alive after 24 hr of O_2 survived until the normal times of death for the untreated rats (i.e. no further deaths occurred between 24 and 48 hr).

The response to O_2 of rats given 250 mg DDC/kg body wt was of special interest. Twenty-six per cent of the animals died in less than 24 hr of O_2 exposure.

Table 1. Effect of diethyldithiocarbamate (DDC) on the response of rats to 95–97% O_2*

	Rat deaths (%)				
	Hours to death				
	1–23	24–47	48–96	> 6-Day survivors	No. of rats
DDC (mg/kg body wt)					
0	0	0	85	15	68
100	0	0	79	21	14
200	0	0	78	22	18
250	26	0	24	50	54
300	59	0	27	14	22
500	84	0	8	8	12

* DDC was injected and the 95–97% O_2 atmosphere was initiated at 0 time; see Materials and Methods for details.

Another 24 per cent died between 48 and 96 hr of O_2 exposure, analogous to untreated animals, but 50 per cent of the rats survived > 6 days of O_2 exposure. The focus of the rest of this study was to determine the reason for the greater number of survivors in the latter group of animals. In particular, we have looked at changes in various O_2 adaptive enzymes in rats injected intraperitoneally with 250 mg DDC/kg body wt and exposed to O_2 for varying times. All enzyme assays were performed on tissue obtained from freshly killed rats. Any rats that died unobserved were discarded; thus, only animals alive at the given times were used for enzyme assays.

Figure 1 shows the changes in G6PD levels in lungs of rats exposed to 95–97% O_2 for various intervals up to 7 days. The G6PD activity of the O_2-exposed control (non-DDC-treated) group was elevated above the pre-exposure level ($P < 0.05$) by 40 hr. The G6PD activities of control animals alive after 69 hr and after 7 days of O_2 exposure were increasingly elevated above the pre-exposure level

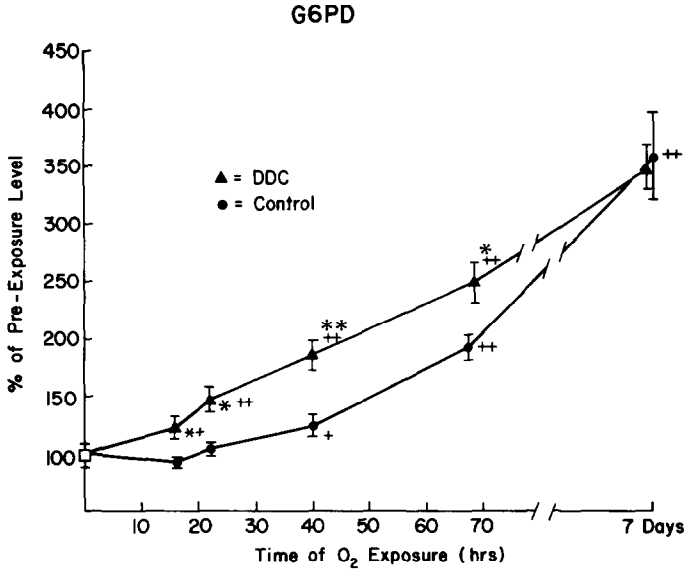

Fig. 1. Effect of 95–97% O_2 on G6PD in control rats and in rats preinjected with 250 mg/kg DDC. Enzyme activity (means ± S.E.M.) is expressed as a percentage of pre-exposure control level. The average value for the pre-exposed (⊡) enzyme activity was 3.02 ± 0.60 (N = 6) μmoles $NADP^+$ reduced/min/pair of lungs at 30°. Key: (*) statistically above control level ($P < 0.05$) (Student's *t*-test); (**) statistically above control level ($P < 0.01$); (+) statistically above initial levels ($P < 0.05$); and (++) statistically above initial levels ($P < 0.01$).

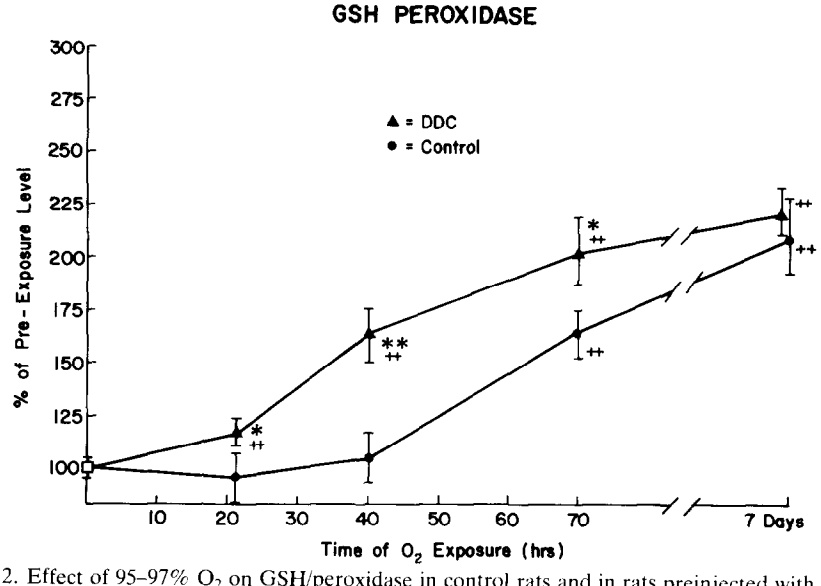

Fig. 2. Effect of 95–97% O_2 on GSH/peroxidase in control rats and in rats preinjected with 250 mg/kg DDC. Enzyme activity (means ± S.E.M.) is expressed as a percentage of pre-exposure control level. The average value for the pre-exposed (⊡) enzyme activity was 1.07 ± 0.06 (N = 7) μmoles NADPH oxidized/min/pair of lungs at pH 7. Key: (*) statistically above control levels ($P < 0.05$) (Student's *t*-test); (**) statistically above control levels ($P < 0.01$); and (++) statistically above initial levels ($P < 0.01$).

($P < 0.01$). Animals pretreated with DDC at 250 mg/kg showed a much more rapid increase in levels of G6PD. G6PD in lungs of these rats was significantly elevated above both initial levels and levels of G6PD in lungs of untreated controls exposed to O_2 alone at 16 hr of O_2 exposure ($P < 0.05$). The elevation above controls of G6PD was maintained at 22, 40 and 69 hr of O_2 exposure. However, the level of G6PD activity was equivalent for animals of both groups that had survived O_2 exposure for 7 days.

Figure 2 shows the results for glutathione peroxidase activities after O_2 exposure. There was no significant increase in peroxidase activity in O_2-exposed controls up to 40 hr of O_2 exposure. Animals alive at 69 hours and 7 days had peroxidase levels which were significantly elevated above pre-exposure levels ($P < 0.01$). The levels of peroxidase in the DDC-treated animals, however, increased much more rapidly with significant changes above controls ($P < 0.05$) and above initial levels ($P < 0.01$) seen as early as 22 hours after O_2 exposure.

Figure 3 shows a similar effect on GSSG reductase activity upon exposure to O_2 with and without 250 mg/kg DDC. The activity of glutathione reductase was higher in O_2-exposed animals after DDC injection than in O_2 controls at 22, 40 and 69 hr of O_2 exposure, with no significant difference between the groups of survivors after 7 days of O_2 exposure.

Levels of SOD activity for O_2-exposed controls and for O_2-exposed animals treated with DDC are shown in Fig. 4 (epinephrine assay). In contrast to G6PD, GSH peroxidase, and GSSG reductase activities, the increase in SOD activity upon O_2 exposure was not accelerated by DDC. There were no significant increases above O_2 control levels at any of the durations of O_2 exposure. However, SOD was significantly elevated above pre-exposure levels for both groups of animals in the 7-day survivors ($P < 0.01$). SOD values were also determined using the cytochrome *c* assay with the same results.

Table 2 shows the effect of O_2 and DDC on SOD levels measured in lung homogenates by both the epinephrine [16] and cytochrome *c* [14] methods. At 40 hr, no statistically significant increase above control levels was seen with either method, and no

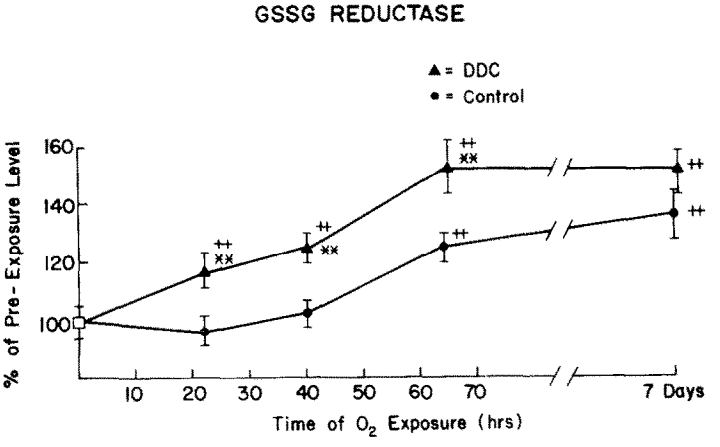

Fig. 3. Effect of 95–97% O_2 of GSSG reductase in control rats and in rats pre-injected with 250 mg/kg DDC. Enzyme activity (means ± S.E.M.) is expressed as a percentage of pre-exposure control level. The average value for the pre-exposed (□) enzyme activity was 2.39 ± 0.28 (N = 7) μmoles $NADP^+$ reduced/min/pair of lungs at pH 7. Key: (**) statistically above control levels ($P < 0.01$) (Student's *t*-test); and (††) statistically above initial levels ($P < 0.01$).

Table 2. Effect of O_2 on superoxide dismutase (SOD) activity determined by the epinephrine and the cytochrome *c* assays*

	SOD (epinephrine assay)†	SOD (cytochrome *c* assay)‡
O time (controls)	608 ± 88 (6)	634 ± 19 (4)
O_2, 40 hr	667 ± 67 (6)	656 ± 41 (4)
O_2 + DDC, 40 hr	655 ± 35 (6)	625 ± 30 (4)

* SOD activity is expressed as units per pair of rat lungs. See Materials and Methods for details. The average value ± S.E.M. is noted. The number of animals studied is in parentheses. None of the values were statistically above control levels by either assay method.

† Misra and Fridovich [16].

‡ McCord and Fridovich [14].

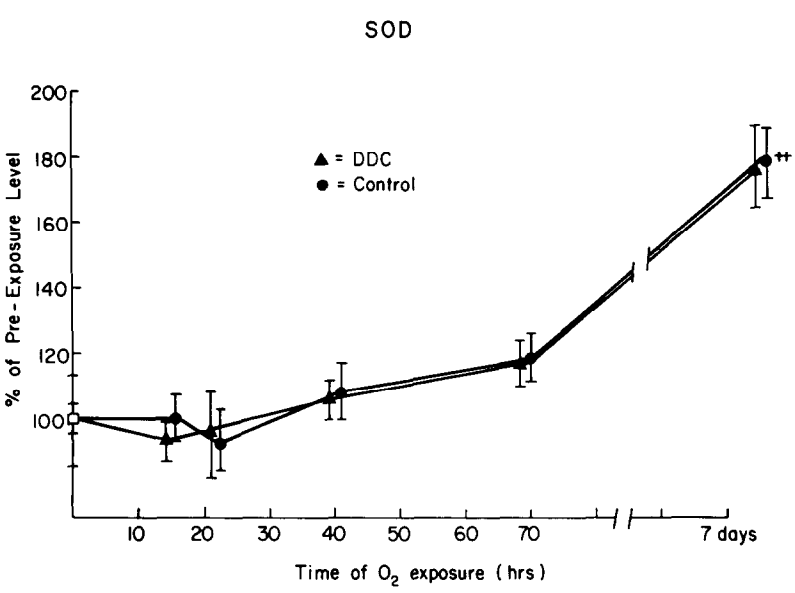

Fig. 4. Effect of 95–97% O_2 on SOD in control rats and in rats pre-injected with 250 mg/kg DDC. Enzyme activities (means ± S.E.M.) are expressed as percentages of pre-exposure control levels. The average value for the pre-exposed (⊔) enzyme activity was 608 ± 88 units/lung (epinephrine assay). Key: (++) statistically above initial levels ($P < 0.01$) (Student's t-test).

increase was seen in the DDC + O_2-exposed animals (250 mg/kg) compared to those rats exposed to O_2 alone.

In order to determine whether the increases in the glutathione enzymes were related, we examined the values of enzyme activity by multiple regression analysis. In the O_2-exposed or O_2 + DDC animals, the increases in G6PD, GSSG reductase and GSH peroxidase are correlated with a multiple correlation coefficient value $R = 0.83$ ($P < 0.005$) for DDC and O_2-treated animals and $R = 0.914$ ($P < 0.002$) for O_2-treated animals. These values were obtained from those animals for which all three enzymes were assayed, using activities at 22, 40 and 69 hr to determine the correlation coefficient. These data are in close agreement with results described by Kimball *et al.* [1] for rats exposed to 90% O_2. For air controls, the correlation coefficient reported was $R = 0.458$ ($P < 0.02$) [1]. The high correlation for the animals which showed elevated levels of the enzymes in response to O_2 or DDC + O_2 suggests that the increase in these enzymes is a concerted process in response to O_2 stress.

Enzyme levels were also measured in lungs of animals injected with 250 mg/kg DDC and maintained in air for various times. These data are shown in Table 3. No increases were seen in GSH peroxidase, GSSG reductase or G6PD at 24 hr of air exposure. At 48 hr there was an apparent slight increase in these enzymes but only the G6PD levels were significantly above initial levels ($P < 0.05$). We have also injected rats with 250–500 mg/kg DDC and exposed these animals to O_2 for 24 or 48 hr after injection. No positive or negative effect of DDC on O_2 survival was seen in these animals, indicating that DDC and significant metabolites are cleared by 24 hr. DDC at levels of 100 and 200 mg/kg has at most a minimal effect on survival of the rats in O_2 (Table 1). Table 4 shows the effects of these lower doses of DDC after 22 hr of O_2 exposure on lung levels of G6PD, GSSG reductase and GSH peroxidase. At 100 mg/kg there was no apparent change from uninjected controls. The levels of these enzymes at 200 mg/kg DDC appear to be somewhat above controls but not significantly elevated and they are less than the levels at 250 mg/kg. At doses of 250 mg/kg

Table 3. Enzyme values after 250 mg/kg diethyldithiocarbamate (DDC) in animals exposed to air*

	GSH peroxidase	GSSG reductase	G6PD
Control (no DDC)	1.07 ± 0.06 (7)	2.39 ± 0.28 (7)	3.02 ± 0.60 (6)
24 hr (+ DDC)	1.12 ± 0.12 (4)	2.36 ± 0.20 (4)	3.24 ± 0.32 (8)
48 hr (+ DDC)	1.20 ± 0.26 (4)	2.68 ± 0.37 (4)	3.82 ± 0.38† (4)

* Values are expressed as total units of activity per pair of rat lungs. Averages ± S.E.M. are noted. The number of rats tested is in parentheses. One unit GSH peroxidase = 1 μmole NADPH oxidized/min at pH 7, 25°. One unit GSSG reductase = 1 μmole NADPH oxidized/min at pH 7, 25°. One unit G6PD = 1 μmole NADPH reduced/min at pH 7.8, 30°.

† Statistically above control ($P < 0.05$).

Table 4. Enzyme levels 22 hr after various doses of diethyldithiocarbamate (DDC) and exposure to 95–97% O_2*

	G6PD	GSSG reductase	GSH peroxidase
DDC (mg/kg body wt)			
0	108 ± 6 (6)	96 ± 5 (6)	97 ± 8 (6)
100	107 ± 10 (6)	97 ± 6 (6)	99 ± 8 (6)
200	115 ± 11 (6)	110 ± 7 (6)	112 ± 7 (6)
250	152 ± 12†,‡ (4)	118 ± 4†,‡ (4)	120 ± 2†,‡ (4)

* Enzyme values are expressed as percentages of pre-exposure untreated controls ± S.E.M.; the number in parentheses equals the number of rats. Significance levels were calculated using the values expressed as enzyme units rather than percentages: 100% G6PD = 3.02 units ± 0.60 (S.E.M.) = 3.02 μmoles NADPH reduced/min; 100% GSSG reductase = 2.39 units ± 0.28 (S.E.M.) = 2.39 ±moles NADPH oxidized/min; and 100% GSH peroxidase = 1.07 units ± 0.06 (S.E.M.) = 1.07 μmoles NADPH oxidized/min. Units expressed are total activity per pair of rat lungs.

† Significantly above pre-exposure untreated rats, $P < 0.05$.

‡ Significantly above 22 hr O_2 controls (without DDC), $P < 0.01$.

at 22 hr, all of the enzyme levels were significantly above control levels ($P < 0.05$).

DISCUSSION

Our results show that the response of rats to hyperoxia after injection of DDC is very different from that previously reported after injection of disulfiram [21]. Disulfiram shortens the survival time of rats exposed to 95–97% O_2. Shortened survival occurs at doses of disulfiram as low as 10 mg/kg body wt. The percentage of early deaths of animals exposed to O_2 increases as doses of disulfiram are increased; all disulfiram-treated rats die in 24–48 hr at doses of 200 mg/kg body wt. By contrast, the acceleration of the death rate in O_2-exposed animals after DDC treatment occurs only after doses of DDC at and above 250 mg/kg body wt. A large number of animals die at 16–22 hr of O_2 exposure when given the higher doses of DDC.

The above observations are consistent with the known chemistry of reaction of the two compounds. In both cases, the free compounds are rapidly metabolized and excreted [11]. Disulfiram, unlike DDC, is capable of binding irreversibly to protein sulfhydryls, thus permanently inactivating enzymes and allowing for longer term effects [22]. DDC is postulated to inhibit various enzymes by chelating Cu^{2+} [12]. This would probably require relatively high local concentrations of DDC and could explain the higher concentration required for toxicity of this compound. The inhibitory effect would most likely be reversible and not persist long after the DDC in the circulation was metabolized. This may explain the observation that DDC causes either very early deaths resulting from hyperoxia or has no effect on the toxicity seen with O_2 alone, since the half-life of the free compound *in vivo* is apparently 2 hr [11]. Disulfiram and DDC are at least partially interconvertible *in vivo* [11, 23], and DDC *in vivo* has been shown to partially inhibit such sulfhydryl enzymes as aldehyde dehydrogenase [23]. Glutathione peroxidase has also been shown to be inhibited by sulfhydryl reagents [24, 25] and has also been shown to be inhibited by DDC *in vivo*. This inhibition may be an indirect effect of a DDC-caused depression of SOD causing elevated O_2^- levels [26] or possibly could be caused by disulfiram, generated by DDC oxidation *in vivo*. The differences observed in the dose-response curves of the two compounds and the time courses of the toxic responses suggest that they do not have a common mechanism of toxicity. Given the difference in lipophilicity of the compounds, one would also expect very different tissue distributions when both were administered intraperitoneally. Thus, the specific mechanism by which each of the compounds accelerates O_2 toxicity is still unknown at this time.

An unusual feature of treatment with DDC is that a protective effect of this compound on rats exposed to hyperoxia occurs at a dose of 250 mg/kg body wt. The protective effect of DDC at threshold toxicity appears to be related to the increases in the various enzymes involved in maintaining an appropriate level of reduced GSH and detoxification of lipid peroxides. These enzymes are significantly elevated in animals exposed to O_2 as early as 22 hr after injection of 250 mg DDC/kg body wt. There are no significant elevations of these enzymes in rats exposed to 250 mg/kg body wt and maintained in air for 24 hr and also no protection against O_2-caused deaths afforded by pre-injection of DDC 24 hr or 48 hr before O_2 exposure. At levels of DDC of 100 and 200 mg/kg body wt there is no significant increase in O_2 survival. In these animals there appears to be a slight elevation of the GSH enzymes above the O_2 control level, but unlike that observed at 250 mg DDC/kg body wt, the increase is not statistically significant after 22 hr.

It has been reported previously that SOD is inhibited in the rat lung by intraperitoneal administration of DDC [13, 21]. However, at levels of 250 mg/kg body wt or less, this enzyme is inhibited 10 per cent or less at 3 or 4 hr after injection of DDC with no significant inhibition by 24 hr after exposure [21]. Thus, it seems unlikely that the toxic response of rat lungs to DDC + O_2 is related to SOD inhibition. The SOD inhibitor effects, however, could be much greater in specific locations in the lung, such as individual cell types or specific subcellular organelles, so that SOD inhibition leading to elevated O_2^- levels is a possible mechanism for the toxic

response to O_2 seen at the higher doses of DDC.

It is clear that both O_2 and DDC are necessary for the early stimulation of enzymatic activity and for the protection against death by O_2 which we have observed. The mechanism of enzymatic stimulation is not known. It was suggested previously that SOD stimulation by elevated O_2^- levels may be mediated by the presence of an excess of O_2^- ions [27]. The glutathione enzymes may also be stimulated by O_2 or other metabolic intermediates of O_2–cell interaction. If the DDC is interfering directly or indirectly with some enzyme system which is responsible for maintaining low concentrations of toxic O_2 metabolites, the addition of O_2 could lead to a burst of active oxidants which might trigger the induction of protective enzyme activity. The lack of stimulation of SOD increase by DDC is seen using both the epinephrine assay (which measures primarily the Cu–Zn SOD) and the cytochrome *c* assay which measures both isozymes [14, 16] (see Table 2). The lack of accelerated response of SOD activity in our system may be for a variety of reasons. One possibility is that DDC interferes with the ability of lungs to increase SOD activity in response to O_2 as was seen in young rats by Frank *et al.* [13]. Another possibility is that enzyme level changes are not directly stimulated by O_2 + DDC but are secondary to changes in cell distribution or function induced by the combination of O_2 + DDC.

We have shown that the combined action of DDC + O_2 causes O_2 tolerance in adult rats and that this tolerance is correlated with increases in G6PD, glutathione reductase and glutathione peroxidase, and that the increases in these enzymes occur in concert. Whether or not specific cellular alterations in the lung contribute to tolerance as well as to increases in these enzymes has yet to be determined.

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