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The data are consistent with the hypothesis that $H_2O_2$ is formed or accumulated in excess in red cells of tocopherol-deficient mice, an effect that is enhanced in the presence of hyperoxia. They imply that tocopherol plays a role in the detoxification of $H_2O_2$.

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# In Vivo Formation of $H_2O_2$ in Red Cells during Exposure to Hyperoxia

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ABSTRACT Chow-fed and tocopherol-deficient mice were given aminotriazole (AT), exposed to 100% $O_2$ at 60 pounds per square inch absolute for 1 hr (OHP), and red blood cells were assayed for catalase activity and lipid peroxide levels. A decrease of catalase activity (CA) in the presence of AT can be taken as evidence of excess formation or accumulation of $H_2O_2$. No differences of CA were observed among chow-fed mice, with or without AT and/or OHP. Tocopherol-deficient mice with AT had lower CA (0.174±0.040) than chow-fed mice with AT (0.225±0.028) $P < 0.01$. Tocopherol-deficient mice with AT exposed to OHP had even lower CA, 0.137±0.024, $P < 0.01$.

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## INTRODUCTION

The toxic effect of 100% oxygen under high pressure (OHP)[1] on red cells (RBC's) has been recognized for several years. Our previous studies showed that: (*a*) Mice deficient in tocopherol (a lipid antioxidant) developed hemolysis during exposure to OHP (1). (*b*) Their in vivo lytic sensitivity to OHP paralleled their in vitro lytic sensitivity to oxidant stress ($H_2O_2$, ultraviolet radiation). (*c*) In vivo hemolysis during OHP was preceded (and presumably caused) by peroxidation of RBC lipid (2). Since various prooxidant drugs had been shown to cause $H_2O_2$ to form in RBC's, in vitro and in vivo (3, 4), the present studies were undertaken to determine if $H_2O_2$ was generated or accumulated in excess in RBC's in vivo during OHP.

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[1] *Abbreviations used in this paper:* AT, aminotriazole; CA, catalase activity, OHP, 100% oxygen under high pressure.

## METHODS

### Mice

Male and female strain DBA/2 mice[2] (9–11 months of age, average weight 25 g) were used for all experiments. A total of 120 (70 experimental, 50 control) animals were used. One group was maintained on a tocopherol-deficient test diet[3] for a minimum period of 6 wk (5). The control animals were maintained on standard chow diets. The weights of mice in each study group did not differ appreciably. Those animals receiving tocopherol supplementation were administered 1 mg of vitamin E[4] i.p. (in 0.1 ml normal saline) 12 hr before exposure to OHP.

### OHP procedure

Exposure to OHP was carried out in a cylindrical hyperbaric chamber[5] with a total volume of 450 cubic inches. Fresh soda lime (USP) was maintained in the chamber to absorb $CO_2$ creating a near 100% oxygen atmosphere. In each experiment, 8–10 animals were placed in the chamber, which was then flushed at normal atmospheric pressure with 100% oxygen for 5 min.

Compression with 100% oxygen to 60 psia (pounds per square inch absolute, 4 atmospheres) was carried out over a period of 15 min in a stepwise manner (15 psia every 5 min). Pressure was maintained for 60 min. Continual circulation of the gaseous environment was maintained throughout the exposure by a constant flow of 10 liters/min of 100% oxygen. 15 min was allowed for slow, stepwise decompression. The total exposure time to 100% oxygen was 95 min. This duration of exposure was chosen because previous studies had established that additional time at this pressure resulted in convulsions and death.

For control studies, the above procedures were repeated substituting gas of room air composition for the 100% oxygen. In all studies, appropriate control mice were maintained at ambient atmospheric conditions and control and study assays were performed simultaneously.

[2] Jackson Laboratories, Bar Harbor, Maine.
[3] General Biochemicals, Chagrin Falls, Ohio.
[4] Aquasol E (aqueous), USV Pharmaceutical Corp., New York.
[5] Table Top Model No. 614, Bethlehem Apparatus Co., Hellertown, Pa.

TABLE I

*Catalase Activities of Tocopherol-Deficient and Chow-Fed Mice*

| Study group | Catalase activity, mEq perborate decomposed/ml hemolystate (0.02 g/100 ml hemoglobin) per 2 min |
|---|---|
| Chow-fed control (25) | 0.230±0.032 |
| Chow-fed + AT (10) | 0.225±0.028 |
| Chow-fed + OHP (10) | 0.217±0.019 |
| Chow-fed + AT + OHP (10) | 0.211±0.016 |
| Tocopherol-deficient control (25) | 0.221±0.033 |
| Tocopherol-deficient + AT (10) | 0.174±0.040* ($P < 0.01$) |
| Tocopherol-deficient + OHP (10) | 0.214±0.043 |
| Tocopherol-deficient + AT + OHP (10) | 0.137±0.024* ($P < 0.005$) |
| Tocopherol-deficient + tocopherol supplemented + AT + OHP (10) | 0.218±0.010 |

All values given represent means ±1 SD.
Figures in parentheses indicate number of animals in each group.
* Indicates groups significantly different from tocopherol-deficient controls.

### Use of aminotriazole (AT) for determination of $H_2O_2$ accumulation and/or generation (3, 4).

Under usual conditions, catalase first forms a complex with $H_2O_2$ which subsequently reacts with another molecule of $H_2O_2$ to form $H_2O$ and $O_2$, and to regenerate free catalase.

When AT is present in the system, it irreversibly binds the intermediary (catalase · $H_2O_2$) complex. Therefore, a decrease in the activity of catalase in a system that includes AT implies the excessive generation or accumulation of $H_2O_2$.

A fresh aminotriazole[6] solution (840 mg/10 cc in 10% sucrose solution) was prepared daily immediately before each experiment and was administered intraperitoneally in a dose of 2100 mg/kg body weight. The experimental animals which received AT were injected 1 hr before and immediately before exposure to OHP. Simultaneous control animals were given identical doses of AT and not exposed to OHP.

### Studies of blood

Within 3–5 min after removal from the chamber, ether-anesthetized mice were exsanguinated by cutting surgically exposed axillary blood vessels. Blood was collected in heparinized pipettes and cooled immediately to 4°C. All determinations were performed on samples from individual mice rather than pooled samples.

*Hematologic parameters.* Hematocrits (by a micromethod) and the degree of hemoglobinuria (Hemastix[7]) were determined on all animals before and after exposure to OHP.

*Lipid peroxide determinations.* Lipid peroxides in RBC's were determined by measuring the pink chromagen (absorbance maximum 535 mμ) formed by the reaction of 2-thiobarbituric acid with a breakdown product of lipid peroxides, malonaldehyde by a method previously described (1).

### Catalase determination

This was performed by a modification of the method described by Feinstein (6). Thrice saline-washed RBC's from heparinized blood samples were lysed in 3 vol of distilled water. 20 μl of the hemolysate was removed and used to determine the hemoglobin content (grams per 100 cc) as cyanmethemoglobin. The hemoglobin content of the lysate was then adjusted to a final concentration of 0.02 g/100 ml, with additional distilled water.

1 ml of this solution was added to 5.0 ml of sodium perborate (0.1 mole, pH 7.0) and 4.0 ml of phosphate buffer (0.1 mole, pH 7.0) and incubated at 37°C for 2 min. The reaction was stopped with 2.0 ml of 8 N $H_2SO_4$. The amount of perborate remaining after incubation with the hemolysate was titrated with standardized 0.1 N $KMnO_4$. The end point of the titration was determined as a persistence of dark pink color for 60 sec. Control blanks using distilled water, with and without perborate instead of hemolysate, were treated as above. Catalase units were expressed as milliequivalents of perborate decomposed per milliliter of hemolysate, per 2 min period.

## RESULTS

The RBC catalase activities of tocopherol-deficient mice and chow-fed mice with and without AT at ambient atmospheric conditions and after exposure to OHP are shown in Table I.

There were no significant differences in the RBC catalase activities between any of the chow-fed groups or between chow-fed and tocopherol-deficient controls.

However, the following significant decreases of catalase activity in the presence of AT (indicating $H_2O_2$ accumulation) were noted: (*a*) Catalase activity of tocopherol-deficient mice with AT was significantly lower ($P < 0.01$) than tocopherol-deficient controls and also significantly lower ($P < 0.01$) than chow-fed mice with AT. (*b*) Catalase activity of tocopherol-deficient mice with AT exposed to OHP, was significantly lower ($P < 0.005$) than controls, and significantly lower ($P < 0.01$) than tocopherol-deficient mice with AT, but not exposed to OHP. (*c*) Tocopherol supplementation of the tocopherol-deficient mice before OHP and AT prevented the above changes.

There were no changes when similar groups of mice were exposed to room air at high pressures.

When tocopherol-deficient mice were exposed to OHP for 60 min, no hemolysis was noted immediately after OHP. After 1 hr, however, significant hemolysis occurred. Table II summarizes some observations in the pre- and posthemolytic phase, after OHP.

These data illustrate the major degree of hemolysis that occurred (Hematocrit 49–29%, with bright red plasma, and marked hemoglobinuria). It was noted that cells before lysis contained larger quantities of lipid peroxides and on the basis of the lower catalase, had formed or accumulated larger quantities of $H_2O_2$. After hemolysis had occurred, the remaining cells had much lower levels of lipid peroxides and higher catalase activity (therefore, lesser $H_2O_2$ formation, or accumulation).

## DISCUSSION

Although the clinical and histopathologic features of oxygen toxicity have been described in detail, the primary mechanism of cell damage by high oxygen tensions

[6] Sigma Chemical Co., St. Louis, Mo.
[7] Ames Co., Elkhart, Ind.

TABLE II
*Comparison of Pre- and Posthemolytic Phases after OHP*

| | Hematocrit | Hemoglobinuria | RBC lipid peroxide Abs. Max. 535 mμ | Catalase activity, mEq perborate decomposed/ml hemolysate (0.02 g/100 ml hemoglobin) per 2 min |
|---|---|---|---|---|
| | % | | | |
| Immediately after OHP (5) before hemolysis | 49±2 | 0 | 0.132±0.006 | 0.128±0.010 |
| 60 min after OHP (5) | 29±4 | ++++ | 0.010±0.002 | 0.171±0.020 |

Values given represent means ±1 SD.
Figures in parentheses indicate number of animals in each group.

has not been elucidated. The development and use of oxygen under high pressure in hyperbaric chambers for medical purposes (7, 8) made these effects of considerable practical importance since the in vivo levels of alveolar, venous, and arterial blood oxygen tensions reached during OHP far exceed those which are achieved by any other means (9).

Work in other laboratories and our own studies had linked OHP, RBC lysis, and lipid peroxidation in vitro and in vivo (10, 11). The present studies establish the fact that $H_2O_2$ is generated or accumulated in excess within the RBC in vivo during exposure of tocopherol-deficient mice to OHP.

It is of particular note that tocopherol-deficient animals with AT at room air conditions had significantly lower catalase levels, which suggests that tocopherol-deficient animals spontaneously generate or accumulate an excess quantity of $H_2O_2$. These observations also suggest that tocopherol plays some role in the detoxification of $H_2O_2$ in vivo. Whether this is the mechanism by which it exerts its lipid antioxidant effect remains to be clarified. However, the fact that immediately after OHP, in tocopherol-deficient mice, those cells which contained the high levels of lipid peroxides also contained high levels of $H_2O_2$ would seem to link these two phenomena. The data also indicate that those cells that form the greatest quantities of $H_2O_2$ are the cells that are subsequently lost during in vivo hemolysis.

If endogenous $H_2O_2$ formation is, in fact, a mediator of $O_2$ toxicity, then the possible potentiating effects of various redox or oxidant drugs (e.g. ascorbic acid, primaquine, etc.) on the clinical susceptibility to the untoward effects of $O_2$ must be considered.

An alternate explanation for the changes observed is that the AT reacted with a catalase-organic (lipid) peroxide complex rather than $H_2O_2$ per se. It is known that catalase can react with organic peroxides as well as $H_2O_2$. This would also link the protective action of tocopherol directly to the peroxidative process which would be consistent with current thinking in this field.

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