# Hydrogen protects auditory hair cells from free radicals

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**Reactive oxygen species (ROS) play a role in the degeneration of auditory hair cells because of aging, noise trauma, or ototoxic drugs. Hydrogenation is a fundamental reduction/deoxidation reaction in living organisms. This study thus examined the potential of hydrogen to protect auditory hair cells from ROS-induced damage. To generate ROS, we applied antimycin A to explant cultures of auditory epithelia, and examined the effect of hydrogen on the protection of hair cells against ROS. Incubation with a hydrogen-saturated medium significantly reduced ROS generation and subsequent lipid peroxidation in the auditory epithelia, leading to increased survival of the hair cells. These findings show the potential of hydrogen to protect auditory hair cells from ROS-induced damage.** *NeuroReport* **20:689–694 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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## Introduction

Hearing disorders affect nearly 10% of the world population. The common causes of sensorineural hearing loss because of cochlear injury are aging, hereditary disorders, noise trauma, and ototoxic drugs. The mechanisms underlying cochlear injury are still not completely known. However, numerous studies have suggested that they involve the production of reactive oxygen species (ROS), which cause cellular injury in the cochlea resulting in sensorineural hearing loss [1–4]. Although they are probably intended to fight against invasive pathogens, ROS seem to produce substantial collateral damage through DNA strand breaks, lipid and protein oxidation [5–7].

Hydrogenation is a fundamental reduction/deoxidation reaction in living organisms. Many reduction processes in the body involve electron transfer from molecular hydrogen. This molecule was recently established as an antioxidant that selectively reduces the hydroxyl radical, and was shown to decrease the cerebral infarction volume after ischemia in rats [8]. Subsequently, protective effects of hydrogen gas have been demonstrated in a mouse model for hepatic injury [9] and in a rat model for myocardial infarction [10]. In the nervous system, hydrogen-rich water was shown to prevent superoxide formation in mouse brain slices [11], and to prevent stress-induced impairments in learning tasks during chronic physical restraint in mice [12]. Moreover, a clinical study showed that consuming hydrogen-rich pure water improves lipid and glucose metabolism in type 2 diabetes patients [13].

The ex-vivo study reported here tested the hypothesis that molecular hydrogen, hydrogen gas, protects against cochlear impairment by scavenging free radicals. We initially generated in situ ROS in the cochlea using an inhibitor of mitochondrial respiratory chain complex III, antimycin A, and showed that they caused direct damage to the hair cells. Then, using a hydrogen (hydrogen gas)-saturated culture media, we demonstrated that hydrogen gas alleviated ROS-induced ototoxicity, suggesting that hydrogen gas has the potential to act as an antioxidant for the treatment of cochlear damage. We also evaluated the generated hydroxyl radicals by fluorescence emission of 2-[6-(4′-hydroxy)phenoxy-3H-xanthen-3-on-9-yl] benzoate (HPF) and lipid peroxidation by immunohistochemistry for 4-hydroxynonenal (HNE).

## Materials and methods

### Animals

The ICR mice (Japan SLC, Hamamatsu, Japan) used in this study were cared for in the Institute of Laboratory Animals of the Kyoto University Graduate School of Medicine. The Animal Research Committee of the Kyoto University Graduate School of Medicine approved all experimental protocols, which were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

### Cochlear explant culture

Postnatal day 2 (P2) ICR mice were deeply anesthetized with diethyl ether and decapitated. The temporal bones were dissected, and the cochleae were freed from the surrounding tissue and placed in 0.01 M phosphate-buffered saline (PBS; pH 7.4). After removing the cochlear lateral wall, the auditory epithelia were dissected from the cochlear modiolus. The tissue samples were then placed on glass-mesh inserts (Falcon, Billerica, Massachusetts, USA) and cultured initially in serum-free

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modified Eagle's medium (MEM; Invitrogen, Eugene, Oregon, USA), supplemented with 3 g/l glucose (Wako Pure Chemicals, Osaka, Japan) and 0.3 g/l penicillin G (Wako), for 24 h at 37°C in a humidified (95%) air: 5% atmospheric $CO_2$. In total, 20 cochlear explants were used in a single culture, and at least three independent cultures were performed for each condition. As the hair cells in the apex are resistant to free radicals [14], the basal turns of the cochlea were used in this study.

### Antimycin A application

The explants were transferred to medium containing antimycin A (Sigma–Aldrich, St Louis, Missouri, USA) at concentrations of 0.1, 1, or 10 μg/ml, with six to nine cochleae incubated at each concentration. The cultures were maintained for 24 h. At the end of the culture period, the samples were fixed for 15 min in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and then provided for immunostaining for myosin VIIa to evaluate the number of surviving hair cells. The specimens were incubated with primary rabbit polyclonal antibodies against myosin VIIa (1 : 500; Proteus Bioscience Inc., Ramona, California, USA). Alexa-Fluor 568 goat anti-rabbit IgG (1 : 200; Invitrogen) was used as the secondary antibody. Specimens were then incubated in Alexa-Fluor 488-conjugated phalloidin (1 : 250; Invitrogen) to label F-actin. The specimens were examined with a Leica TCS-SP2 laser-scanning confocal microscope (Leica Microsystems Inc., Wetzlar, Germany). To quantify the hair-cell loss in the cochlea after the different treatments, inner hair cells (IHCs) and outer hair cells (OHCs) were counted over a 100-μm-long stretch of the auditory epithelia, in two separate regions of the basal turn in each culture (totaling 200 μm). For each treatment, six to nine cultures were evaluated.

### Hydrogen treatment

To assess the efficacy of hydrogen gas for cochlear protection, explants were cultured initially in an airtight box (Chopla Industries, Inazawa, Japan) with reduced-$CO_2$-dependence media, MEM and Leivovitz's L-15 media (Invitrogen) mixed in a 1 : 1 ratio [15], supplemented with 3 g/l glucose and 0.3 g/l penicillin G, at 37°C in humidified (100%) atmospheric air. After 24 h, the medium was changed to one containing antimycin A at a concentration of 0.1, 1 or 10 μg/ml, with or without hydrogen gas for another 24 h. Hydrogen gas was dissolved directly into the media, and a high content of dissolved hydrogen ($1.3 \pm 0.1$ mg/l) was confirmed using a hydrogen electrode (Model M-10B2; Able Corporation, Tokyo, Japan). The pH of the culture media without hydrogen gas was $7.18 \pm 0.02$, and that of the culture media with hydrogen gas was $7.52 \pm 0.02$. The prepared media were used for culture within 30 min. At the end of the experiments, the explants were fixed and provided for histological analysis to evaluate hair-cell survival. Between six and 12 cochleae were used for each condition.

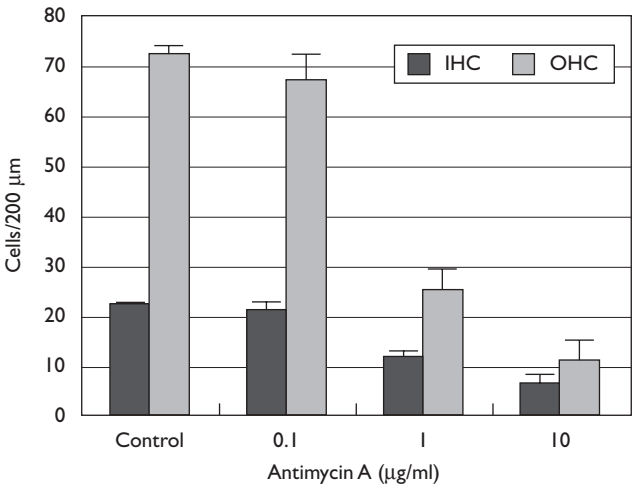
### Detection of reactive oxygen species by fluorescent indicators

The cochlear explants were treated with 30 μM HPF (Daiichi Pure Chemicals Co., Tokyo, Japan) for 20 min to detect cellular hydroxyl radicals. Fluorescent images were captured with a Leica TCS-SP2 confocal microscope using excitation and emission filters of 488 and 510 nm, respectively. All images were taken with the same laser intensity, detector gain, and offset values. Fluorescent signals were quantified from two separate OHC regions of the basal turns, each of which was 1250 $\mu m^2$ ($50 \times 25$ μm), using ImageJ software (*http://rsb.info.nih.gov/ij/*; NIH, Bethesda, MD). Intensity measurements were expressed relative to the levels in the control samples.

### Lipid-peroxidation assay

Lipid peroxidation was assessed in cultures treated with antimycin A, in the presence or absence of hydrogen gas, by measuring the expression of HNE immunohistochemically. Explants were labeled with mouse anti-HNE monoclonal antibody (1 : 8; Oxis Research, Portland, Oregon, USA) and fluorescein horse anti-mouse immunoglobulin G (1 : 250; Vector Laboratories, Burlingame, California, USA) as the primary and secondary antibodies, respectively. Specimens were then counterstained with Alexa 568 phalloidin (1 : 250; Invitrogen). All images were taken with the same exposure and shutter speed. The green fluorescence intensity was measured in the same area using ImageJ software. Intensity measurements were expressed relative to the levels in the control samples.

**Fig. 1**




Antimycin A induced dose-dependent auditory hair-cell loss. The graph shows the relationship between the antimycin A concentration and the hair-cell count following 24-h culture. The inner hair cells (IHCs) and outer hair cells (OHCs) were counted in 200-μm-length regions from each cochlea. The hair-cell densities decreased systematically as the antimycin A concentration increased. Bars represent standard errors.

### Statistical analysis

The overall effects on the hair-cell number, and the HPF and HNE staining intensities, were analyzed by two-way factorial analysis of variance (ANOVA) using the Statcel2 application (OMS Publishing, Saitama, Japan). *P* values less than 0.05 were considered to be statistically significant. For interactions that were found to be significant, multiple paired comparisons were analyzed using the Tukey–Kramer test.

## Results

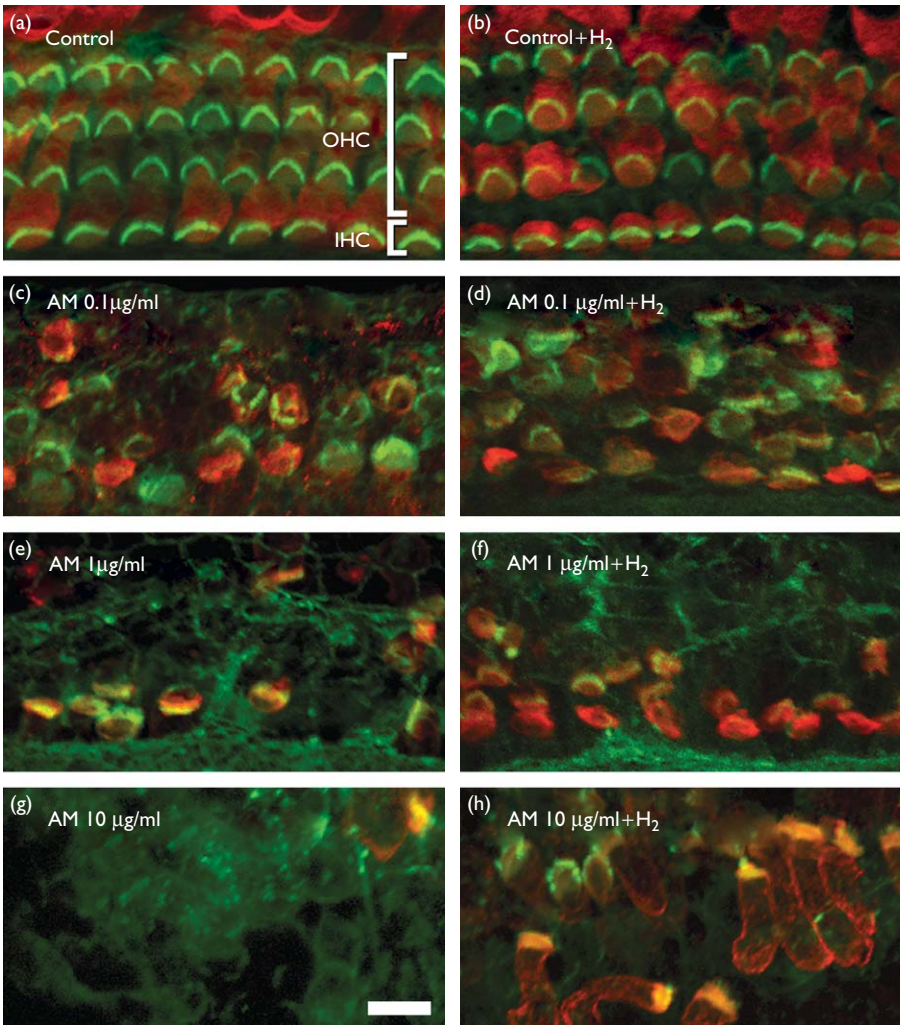
### Antimycin A induced dose-dependent hair-cell loss

Initially, we established the dose–response relationship between the antimycin A concentration and its toxic effect on hair cells. The addition of 1 μg/ml antimycin A to cultures for 24 h significantly reduced the hair-cell numbers in both the IHC and OHC regions, with the effect being more severe in the latter (Fig. 1). The addition of 1 μg/ml antimycin A destroyed 46.2 ± 4.6% of the IHCs and 65.6 ± 5.8% of the OHCs. The hair-cell density decreased depending on the concentrations of antimycin A and few could be detected in the auditory epithelia cultured in 10 μg/ml antimycin A (Fig. 2g).

### Protective effect of hydrogen supplementation

Next, we assessed the potential of hydrogen to protect against antimycin-induced ototoxicity by administering 0, 0.1, 1, or 10 μg/ml antimycin to samples cultured in hydrogen-saturated media in an airtight environment. The addition of hydrogen markedly increased both IHC and OHC survival, with a substantial number of hair cells surviving even at the highest antimycin A dose (Figs 2 and 3). Two-way ANOVA showed that hydrogen gas had a significant effect on the numbers of surviving IHCs and OHCs ($P = 0.00305$ and $P = 0.00016$, respectively). Tukey–Kramer tests for multiple paired comparisons

**Fig. 2**



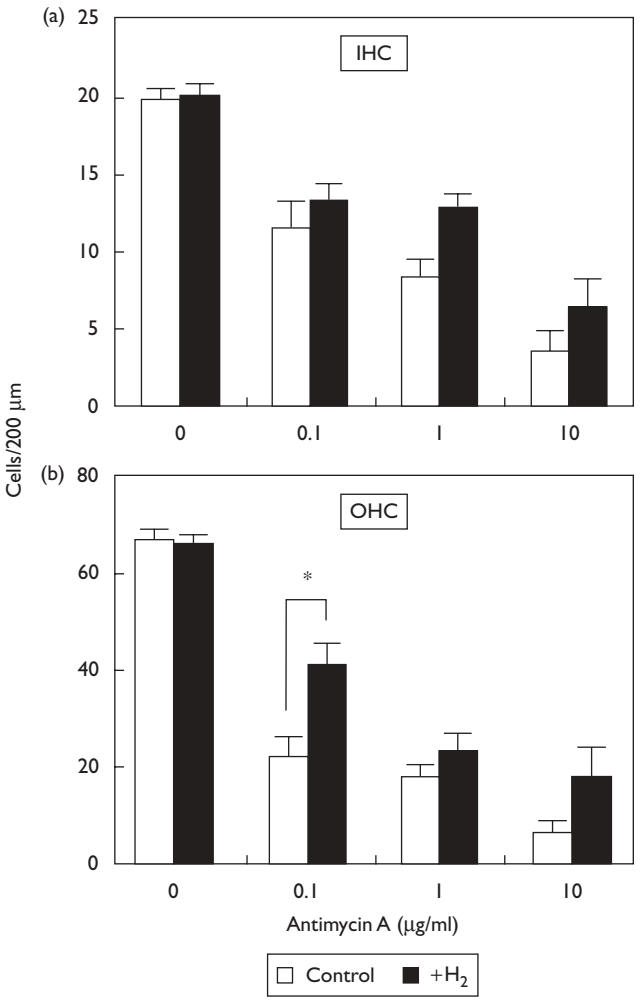

Effect of hydrogen on the survival of cochlear hair cells. (a–h) Photomicrographs of phalloidin (green) and myosin VIIa (red)-labeled cochlear cultures, treated with 0 (a and b), 0.1 (c and d), 1 (e and f), and 10 μg/ml (g and h) antimycin A (AM), with (b, d, f and h) or without (a, c, e and g) hydrogen gas ($H_2$). Bar, 5 μm. IHCs, inner hair cells; OHCs, outer hair cells.

showed that the loss of OHCs was significantly lower ($P < 0.01$) in the groups treated with 0.1 μg/ml antimycin A and hydrogen than in the groups treated with antimycin A alone (Fig. 3). These data showed that hydrogen protected hair cells against antimycin A-induced toxicity in this model of cochlear damage.

**Reactive oxygen species reduction by molecular hydrogen**

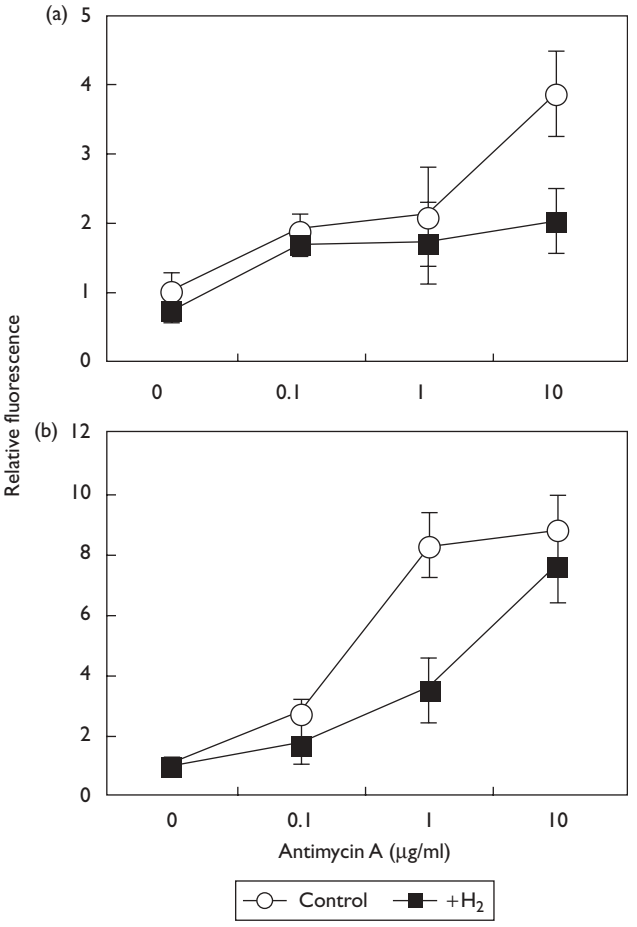
To investigate the mechanism by which hydrogen alleviated hair-cell damage, we measured the ROS production in the cochlear cultures (Fig. 3). HPF is a reagent that was developed to detect certain highly ROS directly [16]. In cochlear cultures treated with 0–10 μg/ml antimycin A for 45 min, in the absence of hydrogen gas, the HPF signals increased (Fig. 4a), indicating that antimycin A induced the production of hydroxyl radicals. The intensity of the HPF fluorescence after the treatment with 10 μg/ml antimycin A was 3.61 times greater than that in the absence of antimycin A (Fig. 4a). By contrast, adding hydrogen gas to the cultures resulted in a reduction of the HPF signal intensity.

**Fig. 3**




Hair-cell counts in molecular hydrogen-treated cultures. After 24-h culture with antimycin A, inner hair cells (IHCs) (a) and outer hair cells (OHCs) (b) were counted. White box symbols represent the counts from control cultures without molecular hydrogen, and black symbols represent those from cultures with molecular hydrogen. Molecular hydrogen significantly attenuated the loss of IHCs ($P=0.0031$) and OHCs ($P=0.0016$) in antimycin A-damaged cochleae according to a two-way analysis of variance. Post hoc analyses with Tukey–Kramer tests for multiple paired comparisons showed that the OHC loss was significantly lower in cultures treated with antimycin A plus hydrogen gas than in those treated with 0.1 μg/ml antimycin A alone (*$P<0.01$). Bars represent standard errors.

**Fig. 4**




Molecular hydrogen reduced reactive oxygen species (ROS) production and lipid oxygenation. White circle symbols represent the counts from control cultures without molecular hydrogen, and black box symbols represent those from cultures with molecular hydrogen. (a) ROS production was measured in cultures treated with antimycin A with or without hydrogen gas for 40 min, according to the intensity of chemifluorescence of 2-[6-(4′-hydroxy)phenoxy-3H-xanthen-3-on-9-yl] benzoate (HPF). The relative HPF fluorescence intensity in the organ of Corti increased dose dependently in the presence of antimycin A, but was significantly reduced by the addition of hydrogen gas ($P=0.0439$). (b) Lipid oxygenation was measured in cultures treated with antimycin A with or without hydrogen gas for 24 h, according to the intensity of immunohistochemical labeling for 4-hydroxynonenal (HNE). The relative HNE-staining intensity in the organ of Corti increased dose dependently in the presence of antimycin A, but was significantly reduced by the addition of hydrogen gas ($P=0.0447$). Bars represent standard errors.

In the presence of hydrogen gas, the intensity of the HPF fluorescence after the treatment with 10 μg/ml antimycin A was 2.34 times greater than that in the absence of antimycin A (i.e. 52.1% of that without hydrogen). Two-way ANOVA showed that hydrogen had a significant effect on the production of hydroxyl radicals ($P = 0.0439$). No significant differences were identified in multiple paired comparisons with Tukey–Kramer tests.

We also investigated the expression of HNE, which is a lipid-peroxidation marker, in the explant cultures. HNE production increased dose dependently in the presence of antimycin A. The intensity of the HNE fluorescence after the treatment with 10 μg/ml antimycin was 8.68 times greater than that in the absence of antimycin A (Fig. 4b). Adding molecular hydrogen to the cultures resulted in a significant reduction of HNE expression. Two-way ANOVA showed that hydrogen had a significant effect on the production of HNE ($P = 0.0446$), but, no significant differences were identified in multiple paired comparisons with Tukey–Kramer tests. Molecular hydrogen had its greatest effect at an antimycin concentration of 1 μg/ml, when the HNE production was attenuated to 42.4% of the level seen in the absence of molecular hydrogen.

## Discussion

To our knowledge, this study was the first to evaluate the therapeutic potential of molecular hydrogen for the auditory system. A quantitative assessment of the hair-cell loss caused by antimycin A showed a dose-dependent effect, indicating that the toxic effects in this explant culture system represented a good model for the cochlea *in vivo*. Treating the cultures with hydrogen gas significantly influenced the dose response for hair-cell loss because of antimycin A, indicating that hydrogen gas has a protective effect on hair cells against ROS toxicity.

We also investigated the mechanisms by which hydrogen gas protected hair cells from damage in the cochlea. Our results showed that the hydrogen gas in fact reduced the production of cellular ROS and subsequent lipid oxygenation. Our antimycin A cochlea culture system, along with chemiluminescence detection, was shown to be useful in screening for antioxidant drugs, because antimycin A directly produces ROS in the cochlea and the direct measurement of ROS was possible when HPF was used.

Hydrogen is one of the most abundant and well-known molecules. Inhalation of hydrogen gas has been used in the prevention of decompression sickness in divers and has shown a good safety profile [8]. Hydrogen has been approved by the US Food and Drug Administration for the treatment of several different diseases. Ohsawa *et al.* [8] demonstrated that hydrogen gas is a potent antioxidant with certain unique properties. First, hydrogen gas is permeable to cell membranes and can target organelles, including mitochondria and nuclei. Second, hydrogen gas specifically quenches detrimental ROS, such as the hydroxyl radical and peroxynitrite, while maintaining the metabolic oxidation–reduction reaction and other less-potent ROS, such as hydrogen peroxide and nitric oxide. The first feature is especially favorable in inner-ear medicine, because many therapeutic compounds are blocked by the blood–cochlear barrier and cannot reach cochlear hair cells [17,18]. Therefore, hydrogen therapy could be widely used in medical applications as a safe and effective antioxidant with minimal side effects.

## Conclusion

In conclusion, this study showed that hydrogen gas markedly decreased oxidative stress by scavenging ROS, and protected cochlear cells and tissues against oxidative stress. These results have prompted us to perform in-vivo studies to determine whether treatment with hydrogen gas might exert a beneficial effect on damaged cochleae and promote hearing recovery.

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