Phosphine-Induced Oxidative Stress in Hepa 1c1c7 Cells

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Phosphine (PH 3), from hydrolysis of metal phosphides, is an important insecticide (aluminum phosphide) and rodenticide (zinc phosphide) and is considered genotoxic and cytotoxic in mammals. This study tests the hypothesis that PH 3-induced genotoxicity and cytotoxicity are associated with oxidative stress by examining liver (Hepa 1c1c7) cells for possible relationships among cell death, increases in reactive oxygen species (ROS) and lipid peroxidation, and elevated 8-hydroxyguanine (8-OH-Gua) in DNA. PH 3 was generated from 0.5 mM magnesium phosphide (Mg 3 P 2) to give 1 mM PH 3 as the nominal and maximal concentration. This level causes 31% cell death at 6 h, measured by lactate dehydrogenase leakage, with appropriate dependence on concentration and time. The intracellular ROS level is elevated within 0.5 h following exposure to PH 3, peaking at 235% of the control by about 1 h. Lipid peroxidation (measured as malondialdehyde plus 4-hydroxyalkenals) is increased up to 504% by PH 3 at 6 h in a time-dependent manner. The level of 8-OH-Gua in DNA, a biomarker of mutagenic oxidative DNA damage analyzed by GC/MS, increases to 259% at 6 h after PH 3 treatment. Antioxidants significantly attenuate the PH 3-induced ROS formation, lipid peroxidation, 8-OH-Gua formation in DNA, and cell death, with the general order for effectiveness of GSH (5 mM) and D-mannitol (10 mM) (hydroxyl radical scavengers), then Tempol (2.5 mM) and sodium azide (3 mM) (superoxide anion and singlet oxygen scavengers, respectively). These studies support the hypothesis that PH 3-induced mutagenic and cytotoxic effects are due to increased ROS levels, probably hydroxyl radicals, initiating oxidative damage. © 1998 Society of Toxicology.

MATERIALS AND METHODS

Phosphine (PH 3), generated by hydrolysis of metal phosphides (AlP, Mg 3 P 2) under ambient environmental conditions (World Health Organization, 1988), is a principal choice for controlling stored-product insect pests (Tomlin, 1997) and is expected to increase in use as methyl bromide is severely restricted worldwide (Taylor, 1996). Zinc phosphide (ZnP 2) is utilized as a bait for rodent control, liberating PH 3 when ingested (Tomlin, 1997). PH 3 is also important as a dopant in the electronics industry (World Health Organization, 1988). Metal phosphides and PH 3 are very toxic to mammals (World Health Organization, 1988). AlP (rat oral LD 50 14 mg/kg) (Batra et al., 1994) is responsible for an epidemic of human poisonings (mostly attempted suicides) in India. Human genotoxicity is evident in fumigant applicators who show increased chromosome rearrangements, primarily translocations in G-banded lymphocytes from peripheral blood (Garry et al., 1989, 1992). Similar genotoxicity from exposure to PH 3 is not observed at occupational levels, by determination of micronuclei in human blood lymphocytes (Barbosa and Bonin, 1994), but subchronic exposure of mice (4.5 ppm, 13 weeks) results in significant increases in micronucleus frequency in bone marrow and spleen lymphocytes (Barbosa et al., 1994).

PH 3 inhibits aerobic respiration with cytochrome c oxidase as the probable target (Chefurka et al., 1976; Price, 1985; Nakakita, 1987; Chaudhry, 1997), an action similar to that of cyanide (Way, 1984). It is proposed that PH 3 in inhibiting cytochrome oxidase in vivo stimulates the production of H 2 O 2 from mitochondria, leading to a "gradual accumulation of oxidant-derived cellular damage, resulting from attack by products of H 2 O 2 reduction" (Bolter and Chefurka, 1990a), such as the very reactive hydroxyl radicals (Bolter and Chefurka, 1990b). PH 3 poisoning also elevates reactive oxygen species (ROS) and causes lipid peroxidation in both insects (Chaudhry and Price, 1992) and humans (Chugh et al., 1996). Although not previously studied, the mechanism for genotoxicity may involve DNA oxidation by ROS.

This investigation uses Hepa 1c1c7 cells to test the hypothesis that PH 3 from metal phosphides elevates ROS levels, leading to lipid peroxidation and oxidative DNA damage as a basis for its genotoxicity and cytotoxicity (Fig. 1).

Chemicals. Sources were as follows: AlP (Chem Service, West Chester, PA) and Mg 3 P 2 (ICN Pharmaceuticals, Inc., Costa Mesa, CA), both >95%.
purity; Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, and phosphate-buffered saline (PBS) from Gibco Life Technologies (Grand Island, NY); 2',7'-dichlorofluorescin diacetate (DCF-DA) from Molecular Probes (Eugene, OR); 8-hydroxyquinoline (8-OH-Gua), 8-hydroxydeoxyguanosine (8-OH-dGua), 7-methylguanine, 7-methylguanosine, and 4-hydroxy-tempo (Tempol) from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

Cell culture. Mouse liver cancer cells (Hepa 1c1c7, Tissue Culture Facility, Department of Molecular and Cell Biology, University of California at Berkeley) were grown in DMEM (pH 7.3) containing 0.37% NaHCO₃, 5% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (culture medium) at 37°C in a humidified atmosphere of 5% CO₂/95% air. Cells (10⁶/10 ml) were added to flasks in 10 ml of medium and incubated for 18 h. The medium was then aspirated and replaced with fresh DMEM (containing no serum and no phenol red) (9 ml). These cultures, treated with test compounds in PBS (1 ml) as below, were capped (to minimize volatilization loss) and incubated at 37°C for 6 h unless indicated otherwise.

Treatment of cell cultures. PH₃ was normally generated from Mg₃P₂ (or AlP when specifically indicated) by adding the metal phosphate to the aqueous medium (Klement, 1963). Typically, Mg₃P₂ was added to PBS at 0.67 mg/ml (5.0 mM Mg₃P₂ or 10 mM PH₃ equivalent), the mixture was capped and shaken, and then the aqueous PH₃ solution was recovered by aspiration after 3–5 min. This clear PBS stock solution (1 ml) was added to DMEM (9 ml) as above to give 0.5 mM Mg₃P₂ or 1 mM PH₃ equivalent; lower concentrations were made by dilution of the freshly prepared stock with PBS immediately before adding to the culture. The PH₃ concentrations stated for these studies are nominal and maximal for several reasons: a saturated aqueous solution of PH₃ is 12 mM (Chaudhry, 1997) and the more PH₃ (or AlP) in preparing the stock solution therefore does not give a higher PH₃ concentration; the conversion of Mg₃P₂ to PH₃ may not be complete as known for AlP in water because of a protective layer of aluminum hydroxide [Al(OH)₃] on the surface (World Health Organization, 1988); some PH₃ may volatilize from the aqueous solution during preparation and assay. Control cells were incubated with either no additive or magnesium hydroxide [Mg(OH)₂] (1.5 mM) or Al(OH)₃ (0.5 mM). In one study, PH₃ was partially removed by degassing the medium with a nitrogen stream.

Antioxidants were evaluated as potential protective agents by addition to DMEM immediately before the PH₃ solution. The test compounds were GSH (5 mM), β-mannitol (10 mM), Tempol (2.5 mM), and sodium azide (3 mM) assayed in the presence of 1.0 mM PH₃ (nominal concentration) or alone (control). Significant differences among multiple groups were determined using a one-way analysis of variance followed by the Bonferroni method to derive p values of <0.05 and <0.01.

Lactate dehydrogenase (LDH) leakage and trypan blue dye exclusion. After agent treatment, aliquots of the media (100 µl) from culture flasks were removed for assay of extracellular LDH activity (Decker and Lohmann-Matthes, 1988). Adherent cells were lysed with Triton X-100 (0.2%) (1 ml) and LDH activity in 100 µl of the cell lysate was also monitored. Cell death was expressed as total LDH activity in the medium + total LDH activity for the medium plus cell lysate × 100, measured colorimetrically using CytoTox 96 (Promega Corp., Madison, WI) for multiplate assays. For trypan blue dye exclusion assays (Freshney, 1994), cells were centrifuged followed by trypsinization. Aliquots of resuspended cells were then stained and counted with a hemocytometer.

Reactive oxygen species. Intracellular ROS were analyzed using DCF-DA, a nonfluorescent, cell-permeable compound that upon entering cells is deesterified and oxidized by cellular ROS to the fluorescent dichlorofluorescein (DCF) (LeBel et al., 1992). The cells were preloaded by incubating them for 30 min at 37°C with DCF-DA (50 µM) in DMEM (10 ml). They were then washed with PBS twice and further incubated with Mg₃P₂ (0.5 mM) or r-butyl hydroperoxide (250 µM) (positive control) for up to 2.5 h. Following treatment, cells were washed twice with PBS and lysed in aqueous Triton X-100 (0.2%) (1 ml) and the fluorescence was directly measured (excitation and emission wavelengths 488 and 525 nm, respectively) with a Perkin–Elmer LS-50B luminescence spectrometer.

Lipid peroxidation. Concentrations of malondialdehyde (MDA) plus 4-hydroxy-2(E)-nonenal (4-HNE), providing a convenient index of lipid peroxidation (Esterbauer and Cheeseman, 1990), were assayed with the Lipid Peroxidation Assay Kit (Calbiochem-Novabiochem Corp. (La Jolla, CA). Briefly, cells (10⁶–10⁷) from an incubation flask were lysed by repeated freeze/thawing in distilled water (1 ml), centrifuged at 15,000g for 10 min to clarify the supernatant, and then treated with N-methyl-2-phenyldimine, which reacts with MDA and 4-HNE at 45°C yielding a stable chromophore, with absorbance determination at 586 nm.

DNA isolation, hydrolysis, derivatization, and GCMS analysis of 8-hydroxyguanine. Purified genomic DNA was isolated by mixing cultured cells (10⁶–10⁷) in buffer with detergent, followed by adsorption to a silica gel-based membrane and elution as specified in the QIAamp Tissue Kit (Qiagen Inc., Chatsworth, CA). Using the procedure of Ravanat et al. (1995), an aliquot of the DNA (50 µg determined by absorbance at 260 nm) was dried under reduced pressure, then 60% formic acid (500 µl) was added for hydrolysis over 30 min at 130°C, followed by solvent evaporation. The residual nucleobases were trimethylsilylated by reaction in a tightly closed screw-cap vial with a mixture of N,O-bis(trimethylsilyl) trifluoroacetamide (25 µl), acetonitrile (15 µl), and pyridine (10 µl) (Pierce, Rockford, IL) at 130°C for 30 min. The derivatized solution (2 µl) at room temperature without further treatment was analyzed by gas chromatography–mass spectrometry (GC-MS) with injection in the splitless mode using a Hewlett-Packard 5890 Series II gas chromatograph coupled to their 5971 Series mass selective detector. HP-1701 column (0.25 mm × 25 mm i.d.) with helium as the carrier gas at 33°C; injector 250°C; temperature program of 60°C for 2 min, then 60°C to 280°C at 20°C/min for 11 min, and finally 280°C for 7 min. The selected ion monitoring mode (methane, chemical ionization) was used to quantitate the MH⁺ ion at m/z 456 and retention time (tR) 12.93 min for derivatized 8-OH-Gua (with four trimethylsilyl substituents) relative to that at m/z 368 and tR 12.80 min for derivatized Gua (with three trimethylsilyl groups). The 8-OH-Gua + Gua peak area ratio on a percentage basis was used as an internal standard in quantitation. The 8-OH-Gua content was determined from a standard curve for the authentic compound.

Reaction of AlP and H₂O₂ with deoxyguanosine to give 8-hydroxydeoxyguanosine. Deoxyguanosine (dGua) (1.0 mg) was treated with AlP (10 mg) and H₂O₂ in a 1:50:500 molar ratio in phosphate buffer (10 mM, pH 7.4, 1 ml) for 6 h at 25°C. Alternatively, guanine (Gua), guanosine (Gus), or other deoxynucleosides (deoxyadenosine, deoxythymidine, and thymidine) were used in place of dGua. The products were analyzed by HPLC which involved a C18 reversed-phase S5 ODS2 column (25 cm × 4.6 mm i.d.) (Phase Separations Inc., Franklin, MA) with a Hewlett-Packard 1050 solvent delivery system coupled to a Hewlett-Packard 1040M Series II diode array detector: 0.1%
trifluoroacetic acid for 5 min, then a linear gradient of 0–75% acetonitrile in water with constant 0.1% trifluoroacetic acid over 25 min, each at 1 ml/min (monitoring at 259 nm for Gua, Guo, and dGuo and 295 nm for their 8-hydroxy derivatives). The rt values (min) were as follows: Gua 11.70, 8-OH-Gua 11.93; Guo 12.85, 8-OH-Guo 13.13; dGuo 12.98, 8-OH-dGuo 13.28. The major product from dGuo with A1P and H2O2 was isolated by HPLC (rt 13.28 min) and subjected to fast atom bombardment (FAB) MS analysis giving MH+ m/z 282. The product from Gua with A1P and H2O2 was analyzed by trimethylsilylation and GC/MS as above.

The reaction of A1P and H2O2 in water or phosphate buffer was varied with other possible reactants (Mg3P2, 2% PH3 gas introduced in nitrogen, phosphinic and phosphonic acids) and oxidants [magnesium monoperphthalate (MMPP) and oxidized GSH (1:50:25 molar ratio dGuo:AlP:GSSG)] with analysis as above.

RESULTS

PH3-Induced Cytotoxicity and Effect of Protective Agents

Treatment of cells with Mg3P2 gives a concentration-dependent increase from 0.1 to 0.5 mM in cell death measured as LDH leakage at 6 h (Fig. 2). Similar cell death at 6 h (33 ± 7%, n = 6) is observed for Mg3P2 and A1P at 1 to 7 mM (which is beyond the saturation level of PH3 in preparing the 10-fold-concentrated stock solution) (data not given). Treatment with 0.5 mM Mg3P2 (nominally 1 mM PH3) results in a time-dependent increase in LDH leakage with 31 ± 4% (n = 3) cell death versus the control value of 8 ± 2% (n = 6) at 6 h (Fig. 3). The toxicant is volatile, as expected for PH3, since the 31% cell death is reduced to 18% when the stock solution is degassed with nitrogen before addition to the cells.

The cytotoxicity of PH3 is markedly reduced by GSH and D-mannitol, less so by Tempol, and not significantly by sodium azide (Fig. 3). In an analogous study with A1P and trypan blue dye exclusion to determine cell death, the 35 ± 6% (n = 6) mortality from PH3 without protective agent was ameliorated as above by D-mannitol and Tempol (data not shown).

PH3-Induced Production of Intracellular ROS and Effect of Protective Agents

Exposure of DCF-DA-preloaded cells to PH3 (from Mg3P2) elevates the intracellular ROS levels evident by 0.5 h (137 ± 30%, n = 3) and reaching a peak level by 1.25 h (235 ± 21%, n = 3), then decreasing to control values by 2.5 h (Fig. 4). In tests with r-butyl hydroperoxide (250 μM) as an exogenous oxidant and positive control, the increase in intracellular ROS at 1 h was 494 ± 54% (n = 3) compared with untreated cells (data not shown). The best protection against PH3-induced production of ROS was afforded by GSH, but Tempol and D-mannitol also significantly lowered the ROS level (Fig. 4).

PH3-Induced Lipid Peroxidation and Effect of Protective Agents

Lipid peroxidation (the level of MDA plus 4-HNE) is significantly elevated to 225 ± 34% (n = 3) by 3 h exposure to PH3 and further increased to 504 ± 124% (n = 3) by 6 h relative to control cells (Fig. 5). Protection is significant with Tempol and highly significant with D-mannitol.
PHOSPHINE-INDUCED OXIDATIVE STRESS

**FIG. 4.** Effect of PH₃ exposure time and candidate protective agents on intracellular ROS level in Hepa 1c1c7 cells measured as DCF-induced fluorescence. Conditions and statistical evaluation for protective agent versus no protective agent (n = 3) at 1.25 h as in Fig. 3. Controls gave 36 ± 2.4 fluorescence units.

**FIG. 5.** Effect of PH₃ exposure time and candidate protective agents on lipid peroxidation in Hepa 1c1c7 cells measured as the sum of MDA and 4-HNE. Conditions and statistical evaluation for protective agent versus no protective agent (n = 3) at 6 h as in Fig. 3. Controls gave 1.4 ± 0.07 nmol (MDA + 4-HNE)/mg protein.

**FIG. 6.** Effect of PH₃ exposure time and candidate protective agents on oxidative DNA damage in Hepa 1c1c7 cells measured as 8-OH-Gua level. Conditions and statistical evaluation for protective agent versus no protective agent (n = 3) at 6 h as in Fig. 3. Controls gave 147 ± 7 (n = 3)/10⁵ bases.

**PH₃-Induced Formation of 8-Hydroxyguanine in DNA and Effect of Protective Agents**

The 8-OH-Gua level in DNA is not changed by treatment with Mg₃P₂ for the first 2 h but is increased to 182 ± 15% (n = 3) and 259 ± 8% (n = 3) of control values by 4 and 6 h, respectively (Fig. 6). GSH and D-mannitol offer more protection than sodium azide, although all three antioxidants provide significant amelioration of the PH₃-induced 8-OH-Gua formation (Fig. 6).

**Reaction of AlP and H₂O₂ with Deoxyguanosine to Give 8-Hydroxydeoxyguanosine**

The reaction of PH₃ (from AlP) and its oxidation products with deoxynucleosides was examined using H₂O₂ as the oxidant. On treatment of dGuo with AlP and H₂O₂ in a 1:50:500 molar ratio, a single major product (20% yield) is evident on HPLC, requiring both AlP and H₂O₂ for its formation, which is not affected by D-mannitol. This product is not formed with AlP or Al(OH)₃ or H₂O₂ alone or with Al(OH)₃ plus H₂O₂. Comparison with an authentic standard characterized the product from dGuo as 8-OH-dGuo (HPLC tᵣ, FAB-MS and λₘᵡₙ 245 and 295 nm). Substitution of H₂O₂ with GSSG (25 molar equivalents) gives a 5% yield of 8-OH-dGuo, but none is observed on replacing AlP in water with Mg₃P₂, 2% PH₃ gas, phosphinic acid, or phosphonic acid or on replacing H₂O₂ with MMPP.

Guo and Gua are converted to 8-OH-Guo and 8-OH-Gua in 5–10% yield with AlP and H₂O₂ as above, but there is little or no reaction with deoxyadenosine, deoxycytidine, thymidine, 7-methylguanine, or 7-methylguanosine, monitored by HPLC at appropriate wavelengths (data not shown).

**DISCUSSION**

Mammalian cell cultures are used here for the first time in studying PH₃ toxicology. The hypothesis was tested that PH₃ from metal phosphides produces oxidative damage to cells, possibly related to previously reported organismal genotoxicity. Hepa 1c1c7 cells (a convenient model system with a population doubling time of about 18 h) are sensitive to PH₃ generated from Mg₃P₂ or AlP with cytotoxicity dependent on concentration and exposure time. With treatment standardized...
at 0.5 mM Mg$_3$P$_2$ (nominally 1 mM PH$_3$) the cultures were sampled at 1–6 h for progressive evaluation of ROS, lipid peroxidation, 8-OH-Gua formation, and cell death in Hepa Iclc7 cells. For conditions see Figs. 3–6. A single line gives the average for observed lipid peroxidation, 8-OH-Gua formation, and cell death because these parameters do not differ significantly from each other. The “% of maximum” is calculated from 0% for the control to 100% for the maximum value observed, which is at 1.25 h for ROS and at 6 h for the other parameters.

FIG. 7. Effect of PH$_3$ exposure time on intracellular ROS level, lipid peroxidation, 8-OH-Gua formation, and cell death in Hepa Iclc7 cells. For conditions see Figs. 3–6. A single line gives the average for observed lipid peroxidation, 8-OH-Gua formation, and cell death because these parameters do not differ significantly from each other. The “% of maximum” is calculated from 0% for the control to 100% for the maximum value observed, which is at 1.25 h for ROS and at 6 h for the other parameters.

DNA oxidation in cells to give 8-OH-Gua could conceivably involve either (a) an oxidant or oxidatively generated reactive phosphorylating species derived from PH$_3$ (Lam et al., 1991) or (b) endogenous ROS induced by PH$_3$. The requirement of oxygen for poisoning (Bond et al., 1967) does not differentiate oxidation of the xenobiotic or generation of endogenous ROS as the mechanism. The former possibility has been studied primarily in model systems. PH$_3$ with m-chloroperoxybenzoic acid and imidazole in chloroform apparently form N-imidazolyl hydrogen phosphonic acid (Lam et al., 1991), which is a possible model for an initial step in 8-OH-dGuo formation. Further studies used a more relevant aqueous system with ALP and H$_2$O$_2$ as the oxidant, chosen since it is known to be released from mitochondria exposed to PH$_3$ (Bolte and Chefurka, 1990), with two interesting observations. First, consistent with the imidazole model above, methylation of Guo and Gua at N7 blocks their ALP/H$_2$O$_2$-induced hydroxylation. Second, and most important, the A1P/H$_2$O$_2$ system converts dGuo to 8-OH-dGuo (Hsu and Casida, 1997). This oxidation was verified with Gua and Gua but did not occur with other deoxynucleosides. dGuo does not react with phosphinic or phosphonic acid directly or with H$_2$O$_2$; therefore, the effective oxidant is possibly PH$_3$(O) (Lam et al., 1991). Similar formation of 8-OH-dGuo from dGuo was shown for GSSG as the

Antioxidant protection helps define the most important ROS contributing to PH$_3$-induced cytotoxicity and genotoxicity (Fig. 1). Hydrogen peroxide is one of the major ROS measured with DCF (LeBel et al., 1992) and further interpretation of the specific ROS involved is based on the specificity of the antioxidants in ameliorating the PH$_3$-induced DCF fluorescence. Tempol lowers the ROS level, suggesting that superoxide anion is involved, i.e., Tempol mimics superoxide dismutase (Kuo et al., 1995). GSH is the most effective antioxidant in lowering PH$_3$-induced ROS, probably by reacting with H$_2$O$_2$ directly or as catalyzed by GSH peroxidases (Farber, 1994). The GSH level in Hepa cells is not changed by PH$_3$ (1 mM nominal concentration) during a period of 6 h exposure and PH$_3$ (from Mg$_3$P$_2$) does not react with GSH or GSSG under physiological conditions within the time scale of these studies (data not given). The alleviating action of GSH for PH$_3$-induced cytotoxicity is therefore not a protection from PH$_3$ directly but instead from a reactive oxidant such as PH$_3$-induced ROS. d-Mannitol, a hydroxyl radical scavenger (Klotz et al., 1997), is not as effective as GSH, perhaps due to hydroxyl radicals acting later in the biological redox system. The PH$_3$-induced effects on lipid peroxidation, 8-OH-Gua in DNA, and cell mortality are generally counteracted more effectively by GSH and d-mannitol than by Tempol or sodium azide (a quencher of singlet oxygen; Klotz et al., 1997), indicating that hydroxyl radicals are the most important agents in the oxidative damage.
oxidant, albeit at lower yield. It is unlikely that hydroxyl radicals mediate oxidation of dGuo with AIP and H₂O₂ since d-mannitol does not affect the yield of 8-OH-dGuo. It is not clear why 8-OH-dGuo formation occurs only with AIP but not with Mg₂P₃ or PH₃ and H₂O₂ or with other oxidants (MMPP), but possible explanations are that only AIP contains Al³⁺ or a contaminant that facilitates formation of PH₃(O) or another reactive intermediate or that the AIP system as a milky suspension differs from the others in potential surface reactions. Nevertheless, formation of 8-OH-dGuo from dGuo by reaction with AIP and H₂O₂ in vitro and 8-OH-Gua in the DNA of cultured Hepa 1c1c7 cells by Mg₂P₃ (probably via ROS) suggests a possible mechanistic relationship to genotoxicity in cases of human poisoning by metal phosphides.

PH₃ is genotoxic in Hepa 1c1c7 cells, evident from the elevation of 8-OH-Gua, which is in itself mutagenic in DNA replication (Wood et al., 1990). 8-OH-Gua is widely accepted as a sensitive marker of oxidative DNA damage (Loft et al., 1993) and in many cases there is a direct correlation between 8-OH-Gua formation and carcinogenesis (Floyd, 1990). Accordingly, monitoring of 8-OH-dGuo in the liver (Lodovici et al., 1997) and urine (Loft et al., 1993) may be a useful guide to the possible relevance of the mechanisms reported here to AIP or PH₃ exposure in animals and humans.

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