Exploring the Toxicology of Depleted Uranium with *Caenorhabditis elegans*

Meiling Lu, Hongyuan Li, Yunfei Li, Yuyuan Lu, Hengshan Wang,* and Xiaohui Wang*

**ABSTRACT:** Depleted uranium (DU) is an emerging heavy metal pollutant with considerable environmental and occupational concerns. Its radiotoxicity is known to be low. However, its chemical toxicity should not be ignored. In order to explore the chemical toxicity of DU, the effects of uranyl nitrate, prepared from DU, on the model organism *Caenorhabditis elegans* were investigated. Chronic exposure to DU did not affect the lifespan or reproduction of the worm. DU had little effect on the physiological processes of *C. elegans*. Additionally, DU treatment did not make *C. elegans* more susceptible to UV, heat, or oxidative stress. Interestingly, chronic exposure of DU decreased the *in vivo* reactive oxygen species-scavenging ability through inhibiting the expression of antioxidant genes *ctl*-1, *ctl*-2, *ctl*-3, *gst*-7, and *gst*-10. Chronic but not acute exposure of DU induced a statistically significant degeneration of the dopaminergic (DAergic) neurons of treated worms and promoted the increase of α-synuclein aggregation and DAergic neurotoxicity. These findings may raise the public concerns regarding DU as an etiologic agent of Parkinson’s disease and underline its potential neurotoxicity.

1. INTRODUCTION

Depleted uranium (DU) is a by-product left over when natural uranium is enriched by increasing the proportion of the isotope 235U for use in nuclear reactors and nuclear weapons.1,2 The major use of DU is in the military as an alloy for armor and ammunition because of its extremely dense and pyrophoric properties.3 Uranium may be released into the environment as it is mined, processed, and applied. While DU is less radioactive than natural uranium, it retains the chemical toxicity of uranium.4,5 As a heavy metal,6 the acute (≤24 h exposure) chemical toxicity of DU is well characterized, and the kidneys are its most vulnerable target.5 However, the chronic (>24 h to several days exposure) DU toxicity has not been well studied, and the findings are not always consistent.5 Whether or not DU causes neurodegenerative diseases is not clear.

In order to investigate the chronic toxicology of DU, herein, the nematode *Caenorhabditis elegans* was chosen as the model organism, which offers several advantages, including a completely sequenced genome,7 freely available numerous multicolor reporter constructs,8 transparent body, a rapid replication cycle, and the ease of growing and maintenance as well as manipulation.9,10 In contrast to rodents which have 10,000–20,000 DAergic neurons, or humans which have greater than 40,000 DAergic neurons,11 *C. elegans* have only eight DAergic neurons: two anterior deirid, four cephalic, and two posterior deirid neurons,12 which makes the in situ investigation of the vulnerability of DAergic neurons upon DU exposure possible.

2. RESULTS

2.1. DU has Little Effect on the Lifespan and Reproduction of *C. elegans*. The nematode, *C. elegans*, is an excellent model organism in the toxicological studies.9,10 First, the lifespan was used as an endpoint to measure the effect of chronic DU exposure on the general health of *C. elegans*. DU concentrations of 0.01 mM (Figure 1A), 0.1 mM (Figure 1B) and 1 mM (Figure 1C) did not affect the lifespan of the wild-type N2 *C. elegans* compared to the untreated control. It should be pointed out that the highest DU concentration (1 mM) tested was relevant to the typical uranium contamination observed in soil (0.2–4.2 mM).13

The effect of DU treatment on the wild-type N2 *C. elegans* reproduction was also investigated. DU did not affect the fertility (Figure 1D). The egg-laying pattern was not notably affected by treatment with 0.01 mM (Figure 1E), 0.1 mM (Figure 1F), and 1 mM (Figure 1G) DU. The egg-laying pattern was not notably affected by treatment with 0.01 mM (Figure 1E), 0.1 mM (Figure 1F), and 1 mM (Figure 1G) DU.

Received: January 27, 2020
Accepted: May 11, 2020
Published: May 19, 2020
The effect of DU on the reproductive system was investigated further. As shown in Figure 1H, no signs of germ cell corpses were observed in the acridine orange (AO) staining, which showed that DU (1 mM) did not induce germ cell apoptosis of worms. Together, these results demonstrate that DU does not affect the lifespan and reproduction of C. elegans.

2.2. DU has Little Effect on the Physiological Processes of C. elegans. To investigate whether DU affects the physiological processes of C. elegans, the locomotive activity and the pharyngeal pumping of the wild-type N2 C. elegans were measured at days 3, 5, 7, and 9, where the L4 stage was defined as day 0. As the animal aged, its head began thrashing, which was quantitatively measured as a body movement, and it progressively decreased with aging (Figure 2A). Because 1 mM DU had little effect on the lifespan of C. elegans after more than 3 weeks of exposure, 0.01 and 0.1 mM DU that are lower than the typical uranium contamination observed in soil (0.2–4.2 mM) were not tested in the following assays. DU (1 mM) treatment did not decrease the body bends of worms at days 3, 5, and 7, while it slightly decreased the body bends of worms at day 9 compared to the untreated control (Figure 2A). Pharyngeal pumping could be counted, and the rate displayed an age-related decline (Figure 2B). DU (1 mM) did not decrease the pharyngeal pumping rate of worms at all time points compared to the untreated control (Figure 2B). Taken together, DU showed little effect on the physiological processes of C. elegans.

2.3. DU has Little Effect on C. elegans’ Response to UV, Heat, and Oxidative Stress. Because DU had little effect on the general health and physiological processes of C. elegans under normal conditions, whether DU (1 mM) could affect C. elegans under stress conditions was subsequently examined. DU treatment did not affect the survival curves of (Figure 1F), or 1 mM (Figure 1G) DU. The effect of DU on the reproductive system was investigated further. As shown in Figure 1H, no signs of germ cell corpses were observed in the acridine orange (AO) staining, which showed that DU (1 mM) did not induce germ cell apoptosis of worms.
the wild-type N2 *C. elegans* upon exposure to UV (Figure 3A), heat stress (Figure 3B), or oxidative stress (Figure 3C) compared to the untreated control. These results indicate that DU does not affect *C. elegans*’ response to various kinds of unfavorable stress.

### 2.4. DU Increase of α-Synuclein Aggregation and DAergic Neurotoxicity

Uranium crosses the blood–brain barrier\(^\text{15}\) and may result in neurotoxicity and neurodegeneration.\(^\text{16}\) Because DU does not affect the general health and the physiological processes of *C. elegans* as well as their responses to unfavorable environmental stress, whether DU (1 mM) could affect the progression of Parkinson’s disease (PD) was tested. The NL5901 strain, which was created by inserting the human α-synuclein gene with an YFP fusion construct driven by the unc-54 promoter, was used as a PD model. As shown in Figure 4A, DU (1 mM) slightly shortened the lifespan of NL5901 worms when compared to the untreated control. NL5901 worms exhibited the aggregation of α-synuclein (Figure 4B). The YFP intensities were quantitatively analyzed and the mean fluorescence intensity in the control group was set to 1. DU (1 mM) treatment did not increase the YFP intensities at days 3 and 5, while it increased the α-synuclein-YFP at day 7 when compared to the untreated control (Figure 4C), which implies that chronic exposure of DU promotes the development of PD.

PD is typically associated with degeneration of DAergic neurons.\(^\text{17}\) Therefore, whether DU (1 mM) could lead to DAergic neurodegeneration was investigated. The BZ555 strain, in which all DAergic neurons are tagged with GFP by fusing with DAT-1, was used to visualize the bodies and processes of DAergic neurons. BZ555 worms were treated with DU (1 mM) and their green fluorescence was analyzed in the nerve ring, which contains GFP-tagged DAergic neurons (Figure 5A). DU (1 mM) treatment did not affect the soma of DAergic neurons when compared to the untreated control at days 1 and 2 (Figure 5B), while it induced a statistically significant shrinkage of the soma of DAergic neurons of treated worms at day 3 (Figure 5B), which indicates that chronic exposure of DU leads to the degeneration of DAergic neurons. To confirm the generality of the results from the transgene BZ555 worms, the effect of DU on the functionality of DAergic neurons was further evaluated by the slowing response assay in wild-type N2 worms. As shown in Figure 5C, DU (1 mM) treatment did not affect the functionality of DAergic neurons at day 1, as revealed by the difference in the average number of body bends per 20 s between N2 worms in OP50-seeded plates and plates without food, while it induced statistically significant inhibition of the functionality of DAergic neurons of the DU-treated worms at days 2 and 3. Together, these results demonstrate that chronic DU exposure induces DAergic neuron degeneration and therefore impairs their functionality.

### 2.5. DU Decreases the Expression of Reactive Oxygen Species-Scavenging Genes

Reactive oxygen species (ROS), which are key modulators in the development of PD,
cause serious damage to and death of DAergic neurons. In order to better understand how DU affects PD development, the effect of DU on in vivo antioxidant genes was investigated. Superoxide dismutases (SODs), glutathione S-transferases (GSTs), and catalases (CTLs) are the main ROS-scavenging enzymes involved in cell detoxification processes. Therefore, the effects of DU on the expression of SODs, GSTs, and CTLs were measured. Chronic exposure of DU decreased the CTL family (ctl-1, ctl-2, and ctl-3) mRNA expression (Figure 6A). In the GST family, DU treatment reduced the mRNA expressions of gst-7 and gst-10, while DU did not affect gst-1, gst-4, and gst-20 gene expression (Figure 6B). In contrast, DU did not affect the SOD family (sod-1, sod-2, sod-3, sod-4, and sod-5) mRNA expression (Figure 6C). These data show that DU decreases the in vivo ROS-scavenging ability by inhibiting the expression of antioxidant genes, which would cause the death of DAergic neurons and promote the PD development.

3. DISCUSSION

Natural uranium consists of three isotopes, $^{238}\text{U}$ (99.27%), $^{235}\text{U}$ (0.72%), and $^{234}\text{U}$ (0.0054%). DU is what remains after the removal of enriched $^{235}\text{U}$, and may also be produced from the reprocessing of spent nuclear reactor fuel. Compared with natural uranium, DU contains a less fissile isotope $^{235}\text{U}$. Therefore, DU has low radioactivity and is not usually considered to exert significant radiotoxicity. In order to systematically characterize the chemical toxicity of uranium, the effects of uranyl nitrate, prepared from DU, on the model organism C. elegans were investigated. DU had little effects on the lifespan, reproduction, and physiological processes of C. elegans under normal conditions. DU treatment did not make C. elegans more susceptible to UV, heat, or oxidative stress, either. They appear to be non-hazardous for C. elegans upon exposure to DU.

Emerging bodies of evidence suggest that heavy metal toxicity promotes the progress of neurodegenerative diseases. PD is characterized by degeneration of dopaminergic neurons containing the aggregated $\alpha$-synuclein protein. Currently, multiple C. elegans PD models, notably the dopaminergic neurons and $\alpha$-synuclein-related transgenic C. elegans PD models, have been used to study the effects of compounds on PD. Uranium is a heavy metal, and the impact of DU on the progression of PD was investigated with C. elegans. Chronic exposure of DU (1 mM) decreased the expression of ROS-scavenging genes, led to the degeneration of DAergic neurons, and promoted the increase of $\alpha$-synuclein aggregation and DAergic neurotoxicity. These results are different from a previous study where Jiang et al reported that...
DU had low neurotoxic potential and did not show any significant neurodegeneration following DU (1 mM) treatment. It should be pointed out that a single end-point (24 h) measurement was made in Jiang et al’s study. Herein, 7 days, but not 3 days or 5 days, of DU (1 mM) treatment increased α-synuclein-YFP in NL5901 worms; 3 days, but not 1 day or 2 days, of DU (1 mM) treatment caused the shrinkage of the soma of DAergic neurons in BZ55 worms; and 2 and 3 days, but not 1 day, of DU (1 mM) treatment impaired the functionality of DAergic neurons in wild-type N2 worms. These were in agreement with Jiang et al’s observation of low acute (24 h) neurotoxic potential of DU. Multigenerational tests of uranium exposure on C. elegans showed rapid phenotypic changes. Most likely, more significant effects on the neuronal damage would be expected for C. elegans after multigenerational exposure to DU, which is worthy of further investigation.

This study found that chronic exposure of uranium caused the degeneration of DAergic neurons, which is consistent with the epidemiologic investigation that the Gulf War veterans exposed to DU had a highly increased prevalence of PD. It should be noted that the sporadic PD is a slowly progressive disorder, which usually takes years/decades to show significant clinical signs. Most likely, the chronic exposure but not the acute toxicology of DU is closely associated with the development of PD.

4. CONCLUSIONS

Overall, this study found that DU did not affect the lifespan and reproduction of C. elegans. Additionally, DU had little effect on the physiological processes of C. elegans. Moreover, DU did not affect the C. elegans’ response to UV, heat, or oxidative stress. However, chronic exposure of DU led to the degeneration of DAergic neurons and promoted the increase of α-synuclein aggregation and DAergic neurotoxicity. These findings may raise public concerns regarding DU as an etiologic agent of PD and underline its potential neurotoxicity. However, all these data are only suggestive until they can be tied to meaningful human research. Therefore, the effects of chronic DU exposure on the central nervous system and neurodegenerative diseases warrant further exploration.

5. EXPERIMENTAL SECTION

5.1. Chemicals. DU (UO2(NO3)2·6H2O, ≥99.9%) was purchased from Chushengwei Chemical Co., Ltd. (Wuhan, Hubei, China). The radioactivity of UO2(NO3)2·6H2O was checked with a Geiger counter to be in the range of 0.2–0.3 μSv/h, which was not significantly different from the in-house background radioactivity.

5.2. Worm Strains, Culture, and Synchronization. The worms were grown on a solid nematode growth medium (NGM) plate in an incubator. To synchronize, 50 worms at day 1 of the postadult period were picked in a new NGM plate and incubated for 6 h at 20 °C to lay eggs. The following strains were used in this work: Bristol strain N2 was used as the wild-type N2 [unc-54p::alpha synuclein::YFP + unc-119(+)], BZ55 [dat-1p::GFP]. All the strains were obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota. All strains were grown and incubated at 20 °C on NGM plates with Escherichia coli (E.coli) OP50.

5.3. Lifespan Measurement. Healthy worms at the young adult stage were placed on NGM plates with E. coli OP50 and different concentrations of DU. To prevent the production of offspring, 5-fluorodeoxyuridine was added. Each group contained 50 nematodes. Worms were transferred to new plates every two days. The number of survivors was recorded daily until all the worms died. The lifespan is defined as the time period between the young adult stage and death. The experiment was repeated three times independently.

5.4. Reproduction Assay. For reproduction assay, age-synchronized L1 stage nematodes were transferred to fresh plates, which was followed by DU exposure. At the L4 larval stage, the parent worm was transferred to a fresh exposure plate every 12 h and the number of offspring at all stages is counted and compared with the control group. The experiment was independently repeated three times for the reproduction test. Thirty worms were examined with three replicates.

5.5. AO Staining. Germ cell corpses were measured by AO staining. Age-synchronized L1 stage nematodes were transferred to fresh plates, which was followed by DU exposure. At day 1 postadult stage, the treated worms were stained for 1 h in the dark at 20 °C by transferring worms into a plate containing 500 μL of 25 μg/mL AO and OP50 in M9 buffer and then transferred to the NGM plate for 40 min for recovery on bacterial lawns.

Worms were then mounted onto 2% agarose pads and immobilized with 2 mM levamisole. To monitor the germ cell, worms were microscopically visualized and photographed using a fluorescence microscope. The apoptotic cells appeared yellow or yellow-orange after AO staining, representing increased DNA fragmentation, while intact cells were uniformly green in color.

5.6. Locomotion and Feeding Behavior Assay. Locomotion behavior was quantified by monitoring body thrashing. To assess body thrashing, the wild-type C. elegans (N2) were incubated for 6 h at 20 °C and allowed to lay eggs, then the synchronized worms at the L4 larval stage were treated with or without 1 mM DU. Worms at days 3, 5, 7, and 9 after the adult stage treated with or without DU were washed with M9 buffer and subsequently transferred to a slide glass containing 100 μL of M9 buffer. After 1 min recovery, body thrashes were counted for 1 min. A movement of the worm that swings its head and/or tail to the same side is counted as one thrash. Thirty worms were examined with three replicates. Feeding behavior was evaluated by the pharyngeal pumping rate, which was measured by counting the number of pharyngeal contractions for 30 s. The test was repeated three times with 10 randomly selected worms per treatment.

5.7. Stress Resistance Assays. To assess the stress resistance in solid culture medium, the wild-type N2 worms were incubated for 6 h at 20 °C and allowed to lay eggs, then the synchronized worms at the L4 larval stage were treated with DU until day 3 postadult stage. For oxidative stress, worms from each group were next transferred to plates containing 200 μM juglone (Sigma-Aldrich, St. Louis, MO, USA) in a NGM plate. To assess UV irradiation resistance, worms from each group were exposed to UV irradiation (254 nm) at a dose of 1000 J/m2 for 30 min and immediately transferred to an incubator set to 20 °C. To assess the heat shock resistance, the worms were transferred from an incubator set to 20 °C to the one set to 35 °C. The viable nematodes were scored every hour until all animals died. At least 150 worms were examined with three replicates.
5.8. α-Synuclein Aggregation Measurement. A transgenic strain, N2, was exposed to different concentrations of DU from the egg stage to day 1, 2, and 3 postadult stage on OP50 plates. At the end of exposure, worms were mounted onto 2% agarose pads and imaged with a Nikon Ts2-FL fluorescence microscope. At least 30 worms were examined with three replicates were imaged, and the fluorescent signals were quantified in each worm using ImageJ software.

5.9. DAergic Neurodegeneration Measurement. A transgenic strain, BZ555, which expresses GFP in DAergic neurons through the dat-1::GFP reporter system, was used to assess the effect of DU on DAergic neurons; the worms were exposed to different concentrations of DU from the egg stage to day 1, 2, and 3 postadult stage on OP50 plates. At the end of exposure, worms were mounted onto 2% agarose pads and imaged with a Nikon Ts2-FL fluorescence microscope. DAergic neurons were counted by inspecting the GFP fluorescence, which could be quantified with ImageJ software. At least 20 worms were examined with two replicates.

5.10. Basal Slowing Response. This assay was used to assess the effect of DU on the dopaminergic neuronal function. N2 worms were exposed to different concentrations of DU from the egg stage to day 1, 2, and 3 postadult stage on OP50 plates. At the end of exposure, worms were collected by washing the plate with M9 buffer; ten worms were transferred to OP50-seeded plates with ring-shaped OP50 lawn or to unseeded plates. After 5 min, the number of body bends was counted to assess the locomotor rate on OP50-seeded or unseeded plates. 6-OHDA (6-hydroxydopamine)-treated N2 worms, whose DAergic neurons were injured, were used as positive control.

5.11. qRT-PCR. Wild-type C. elegans (N2) were incubated for 6 h at 20 °C and allowed to lay eggs, then the synchronized worms at the L1 larval stage were treated with or without 1 mM DU. Then, approximately 1000 worms were suspended in 1 mL M9 buffer, and the worms were washed with M9 buffer 3 times. Total RNAs from worms were prepared by using the TRIzol Reagent kit (Takara Bio, Kusatsu, Japan) according to the manufacturer’s instructions. The cDNA was generated with oligo(dT) primers (Promega, Madison, WI, USA) by using the reverse transcription system (Promega, Madison, WI, USA). The quantitative real-time PCR (qRT-PCR) was carried out using the SYBR Green real-time PCR master mix (Roche, Basel, Switzerland) on a TOPTical Real-Time PCR system (Analytik Jena AG, Analytik Jena, Germany). The mRNA expression levels of genes were normalized to actin-1. The primer sequences for PCR are shown in Table 1.

5.12. Statistical Analysis. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Data were presented as mean ± SEM. For qRT-PCR assays, statistical analysis was done by Student's t-test. For stress resistance and lifespan assays, statistics was analyzed by the log-rank test. For the α-synuclein and DAergic neuron fluorescence images, fluorescence intensities were quantified using ImageJ software, and the statistics was performed by Student’s t-test.

### Table 1. Primers Used in qRT-PCR

| Primers   | Sequences (5’-3’) |
|-----------|-------------------|
| gcs-1 forward | atctcggagagaggacgctgctgaacgtaagttacgtctgaagttgacagctgaagtcagcataatcttttttcttttttcttttttttctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
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Notes

The authors declare no competing financial interest.

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Acknowledgments

This work was supported by the Chinese Academy of Sciences (CAS) Pioneer Hundred Talents Program. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

ACKNOWLEDGMENTS

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