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Mechanism of the Lifespan Extension of *Caenorhabditis elegans* by Electrolyzed Reduced Water—Participation of Pt Nanoparticles

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Electrolyzed reduced water (ERW) contains a large amount of molecular hydrogen and a small amount of Pt nanoparticles (Pt NPs). We have found that ERW significantly extended the lifespan of *Caenorhabditis elegans* in a novel culture medium designated Water Medium. In this study, we found that synthetic Pt NPs at ppb levels significantly extended the nematode lifespan and scavenged reactive oxygen species (ROS) in the nematode induced by paraquat treatment. In contrast, a high concentration of dissolved molecular hydrogen had no significant effect on the lifespan of the nematode. These findings suggest that the Pt NPs in ERW, rather than the molecular hydrogen, extend the longevity of the nematode, at least partly by scavenging ROS.

Key words: electrolyzed reduced water; *Caenorhabditis elegans*; lifespan; reactive oxygen species; Pt nanoparticles

Electrolysis of water produces electrolyzed reduced water (ERW) at the cathode site. ERW exhibits a high pH, low dissolved oxygen, high dissolved molecular hydrogen, and negative redox potential values that vary from -200 to -800 mV.[1)] The Ministry of Health, Labor, and Welfare of Japan recognizes potable ERW as effective for the improvement of gastrointestinal symptoms in the Pharmaceutical Affairs Law of Japan, based on the results of randomized double-blind clinical trials.[2)] Recently, ERW has attracted much attention in view of its antioxidant potential. ERW scavenged reactive oxygen species (ROS) *in vitro* and protected DNA against oxidative damage.[1)] It suppressed the oxidative damage induced in pancreatic β HIT-T15 cells by alloxan, a type 1 diabetes inducer.[3)] It suppressed the angiogenesis of tumor cells.[4)] It showed a significant anti-diabetic effect in diabetic model mice,[5,6)] and an anti-hangover effect in Sprague-Dawley rats.[7)] Therapeutic effects of ERW have also been reported for hemodialysis-induced oxidative stress in renal disease patients.[8–10)]

Excess oxidative stress is known to cause aging and a short lifespan in various animals.[11)] Recently, we found that ERW significantly extended the lifespan of wild-type *Caenorhabditis elegans* in a novel medium designated Water Medium and suppressed the level of reactive oxygen species (ROS) in *C. elegans* induced by paraquat, a oxidative stress-inducing agent.[12)] TI-200 ERW was produced by electrolysis of 2 mM NaOH using a TI-200 batch-type electrolysis device. Despite its simple composition, TI-200 ERW had effects in lifespan extension of *C. elegans* very similar to potable TI-9000 ERW, which was produced from tap water using a continuous TI-9000 electrolysis device for home use. These findings suggest that TI-200 ERW is a useful model for investigating the mechanisms of the therapeutic effects of ERW.[12)]

Since the electrolysis of water produces molecular hydrogen on the cathode, freshly produced ERW always contains a high concentration of dissolved hydrogen. It has been reported that molecular hydrogen selectively scavenges hydroxyl radicals and peroxynitrite, and that inhalation of 2% hydrogen gas can improve the symptoms of cerebral infarction model rats.[13)] It has also been reported that molecular hydrogen suppresses superoxide anion radical formation in brain slices from mice,[14)] and improves the state of oxidative stress-related disease model animals.[15–19)]

On the other hand, we have reported that TI-200 ERW contains ppb levels of Pt nanoparticles (Pt NPs), which are assumed to become detached from Pt-coated titanium electrodes during the electrolysis process.[12)] We have found that synthetic Pt NPs with average sizes of 1–6 nm exhibit superoxide dismutase- and catalase-like activities as well as hydroxyl radical-scavenging activities and protect cultured cells against oxidative stress.[20)] It has also been reported that Pt NPs are a newly recognized kind of antioxidant, and that 0.5 mM Pt NPs can significantly extend the lifespan of *C. elegans* in an S Medium environment.[21)]

In the present study, we investigated the mechanism of the lifespan-extending capability of ERW using Water Medium.

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Materials and Methods

C. elegans *strain and growth conditions.* Wild-type N2 *C. elegans* was kindly provided by Dr. Kazuya Nomura (Department of Biology, Faculty of Sciences, Kyushu University) and maintained at 20 °C following procedures established by Brenner.[22] Age-synchronized populations were prepared as described by Emmons *et al.*[23] Briefly, gravid hermaphrodites were washed from NGM agar and lysed in lysis solution comprising 0.1 mL of 10 M NaOH, 0.5 mL of household bleach (5% solution of sodium hypochlorite), and 5 mL of S basal solution (100 mM NaCl, 0.01 mM cholesterol, and 50 mM potassium phosphate pH 6.0).[24] The collected eggs were hatched overnight, and the resulting first-stage larvae (L1) were transferred to fresh NGM agar plates containing a lawn of *Escherichia coli* OP50 as food source and incubated at 20 °C until the L4 larval stage.

Preparation of ERW. ERW was produced using a batch-type electrolysis device (Type TI-200; Nihon Trim, Osaka, Japan). Briefly, ultrapure water (MQ water) produced by a filtration system (Millipore, Billerica, MA) was supplemented with 2 mM NaOH and electrolyzed for 1 h at a constant voltage of 100 V to produce TI-200 ERW.

Preparation of Pt NPs. Pt NPs with an average size of 2.5 ± 1 nm were synthesized by a previously reported sodium citrate reduction method.[25] Briefly, a total reaction mixture of 300 mL containing 636 μL of 250 mg/mL H_2PtCl_6, 290 mL of MQ water, and 10 mL of 3% sodium citrate was boiled for 2 h under an atmosphere of N_2 gas. After the mixture turned deep black, the reaction was terminated by cooling on ice. Subsequently, the synthesized Pt NPsolution was desalted by adding 10 volumes of MQ water to 1 volume of Pt NPs solution and concentrated by ultrafiltration with a 10,000 molecular weight cut-off ultrafiltration membrane (Advantec MFS, Dublin, CA). The desalting and concentration steps were repeated 3 times. The concentration of Pt NPs was determined by inductively coupled plasma mass spectrometry (Model 7500; Agilent Technologies, Palo Alto, CA) at the Center for Advanced Instrumental Analysis, Kyushu University. The concentration of the synthesized Pt NPs was adjusted to 200 mg Pt/L, and the Pt NPs solution was stored at 4 °C until use.

Preparation of Water Medium. Water Medium was used to examine the effects of water samples on *C. elegans*.[12] It consisted of water samples with the pH adjusted to 7.0 by HEPES (Wako Pure Chemicals, Tokyo) using a pH meter (Model f-32; Beckman-Coulter, Fullerton, CA). Since the pH of TI-200 ERW was 11.6, NaOH solution with the pH neutralized from 11.6 to 7.0 was used as control to make the osmotic value of the treatment samples equal to that of the ERW samples. The final HEPES concentration was about 16 mM.

To avoid any influence of living bacteria, 2×10^9 bacterial cells/mL of heat-killed *E. coli* OP50 (65 °C, 12 h) was used as the nematode food. 5-Fluoro-2′-deoxyuridine (50 μM) was added to the media during the assays to prevent worm death from internal hatching.[26] The dishes were shaken in a gyratory shaker at 120 oscillations/min[26] to supply sufficient oxygen to the worms.

Lifespan assays. For lifespan assays, L4 larvae were transferred to each culture medium[27] and cultured at 20 °C. As described previously,[12] almost all of the dissolved molecular hydrogen was lost during pH adjustment to 7.0 for the preparation of ERW-Water Medium. To examine the effect of dissolved molecular hydrogen on the nematode lifespan, the worms were cultured in 35-mm Petri dishes that were sealed in a plastic box ($L \times W \times H$: $20 \times 14 \times 10$ cm) under an atmosphere of 80% hydrogen/20% oxygen. The worms in the plastic box were cultured under the same conditions as the others. To exclude the effect of the dissolved hydrogen in ERW, the worms were cultured in pre-vacuumed ERW-Water Medium.

The transfer day was designated day 1. The worms were transferred to fresh culture medium using a platinum wire every second day. During this process, they were considered dead if they did not respond to repeated prodding with a platinum wire, and mortality was scored. Worms that crawled away, had internally hatched larvae, or had eviscerated gonads were excluded. A small proportion of the worms died abnormally during the first transfer, and these were also excluded. The mortality data were derived from Kaplan–Meier survival curves,[26] and statistical comparisons of mean lifespans between the worms in the control and ERW groups were performed by log-rank test[26] using the StatMate III Excel add-in program (ATMS, Tokyo).

ROS detection. Young adult worms were cultured in Water Medium containing 0, 2, 5, or 10 ppb Pt NPs until day 5. On day 5, the worms were washed extensively with MQ(NaOH)-Water Medium and incubated for 5 h in 0.2 M 1,10-dimethyl-4,40-bipyridinium dichloride (paraquat; Supelco, Bellefonte, PA), a model of oxidant-initiated toxicity widely used to catalyze the formation of ROS and induce oxidative stress.[28] Subsequently, the worms were washed 3 times with MQ(NaOH)-Water Medium. After the mortality of the worms was counted, all worms were transferred to 2 mL of MQ(NaOH)-Water Medium containing 10 μM dichlorofluorescein diacetate (DCFH-DA; Invitrogen, Carlsbad, CA) and incubated for 30 min at 20 °C. Finally, they were fixed with 4% formaldehyde. The fixed worms were examined by laser scanning confocal microscopy (OLFV-32U2/XE; Olympus, Tokyo) with excitation at 488 nm and emission at 510 nm[29] on the second day. The fluorescence intensities of the worms were determined using FV10-ASW 1.4 software (Olympus). The statistical significance of differences between the control and treated groups was determined by Newman-Keuls test or Welch test.

Results

Effects of dissolved hydrogen on the nematode lifespan

As shown in Fig. 1, the survival curve for N2 *C. elegans* in TI-200 ERW-Water Medium was in good accordance with that in our previous study.[12] The worms cultured in TI-200 ERW-Water Medium showed a 16% longer lifespan than the control worms cultured in MQ-Water Medium, and the difference was significant ($p < 0.001$), but there was no significant difference in lifespan, between the control worms and the worms cultured in an atmosphere of 80% H_2/20% O_2. On the other hand, the worms cultured in vacuumed TI-200 ERW showed a 12% longer lifespan than the control worms, and this difference was also significant ($p < 0.05$). No significant difference in lifespan was found between the worms cultured in TI-200 ERW-Water Medium and those were cultured in vacuumed TI-200 ERW-Water Medium.

Effects of Pt NPs on the nematode lifespan

Previous transmission electron microscope analyses revealed that the Pt NPs in ERW have diameters of about 1–6 nm and concentrations of 0.1–2.5 ppb.[12] In the present study, synthesized Pt NPs at various concentrations were used to examine the effects of Pt

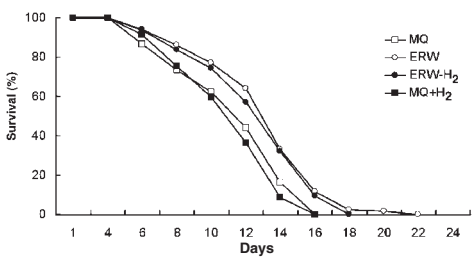

Fig. 1. Effects of Dissolved Hydrogen on the Nematode Lifespan.
Hollow squares, survival curve for nematodes cultured in MQ-Water Medium; hollow circles, survival curve for nematodes cultured in TI-200 ERW-Water Medium; solid circles, survival curve for nematodes cultured in vacuumed TI-200 ERW-Water Medium; solid squares, survival curve for nematodes cultured in MQ-Water Medium in an 80% hydrogen/20% oxygen atmosphere.

Table 1. Effects of Hydrogen and Pt NPs on the Lifespan of *C. elegans*

Trial	Treatment	MLS (d)	MLS (%)	Max (d)	*p*	*n*
1	MQ(NaOH) Water medium	11.6 ± 0.03	100	16		98
	H_2 Water medium	11.4 ± 0.03	98	16	—	102
	ERW Water medium	13.4 ± 0.03	116	22	<0.001	130
	pre-vacuumed ERW medium	13.0 ± 0.03	112	18	<0.001	128
2	MQ(NaOH) Water medium	9.9 ± 0.03	100	18		111
	H_2 Water medium	10.0 ± 0.03	101	18	—	107
	ERW Water medium	13.1 ± 0.04	132	24	<0.001	128
	pre-vacuumed ERW medium	13.6 ± 0.03	137	22	<0.001	118
3	MQ(NaOH) Water medium	12.1 ± 0.02	100	23		185
	2 ppb Pt Water medium	14.3 ± 0.03	118	25	<0.001	136
	10 ppb Pt Water medium	12.7 ± 0.03	105	23	—	149
	50 ppb Pt Water medium	12.3 ± 0.05	102	23	—	148
4	MQ(NaOH) Water medium	15.5 ± 0.03	100	23		135
	2 ppb Pt Water medium	16.1 ± 0.03	105	25	—	139
	5 ppb Pt Water medium	16.6 ± 0.03	107	25	<0.05	130

MLS, mean lifespan, presented as the mean ± SEM (d); %, change in the mean lifespan as compared with control; Max, maximum lifespan (d); *p*, comparison with untreated animals by log-rank test; *n*, number of nematodes examined

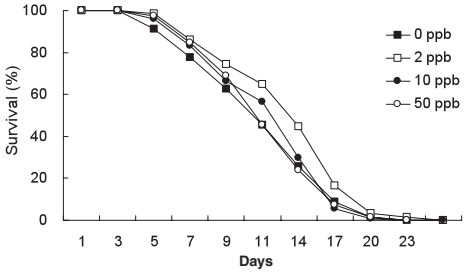

Fig. 2. Effects of Pt NPs on the Nematode Lifespan.

Solid squares, survival curve for nematodes cultured in Water Medium; hollow squares, survival curve for nematodes cultured in Water Medium supplemented with 2 ppb Pt NPs; solid circles, survival curve for nematodes cultured in Water Medium supplemented with 10 ppb Pt NPs; hollow circles, survival curve for nematodes cultured in Water Medium supplemented with 50 ppb Pt NPs.

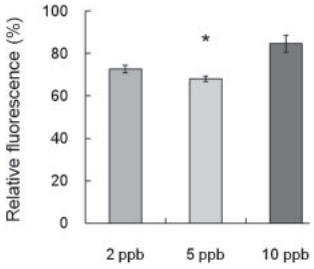

Fig. 3. Effects of Pt NPs on Paraquat-Induced ROS Accumulation in *C. elegans*.

Worms were cultured in Water Medium supplemented with 0, 2, 5, or 10 ppb of Pt NPs for 5 d and then treated with 0.2 M paraquat for 5 h ($n = 10$). The amounts of peroxides in the nematodes were detected by DCFH-DA staining. The average DCFH-DA fluorescence intensity of the nematodes cultured with 0 ppb Pt NPs-Water Medium was normalized to 100%. The mean relative fluorescence intensities of the worms are presented as means ± SEM in comparison with those of the worms cultured in 0 ppb Pt NPs-Water Medium. The labels 2 ppb, 5 ppb, and 10 ppb refer to the relative fluorescence intensities of *C. elegans* cultured in Water Medium supplemented with 2, 5, and 10 ppb Pt NPs for 5 d respectively and then treated with 0.2 M paraquat for 5 h. *p*, comparison with paraquat-treated animals in 0 ppb NPs-Water medium by the Newman-Keuls test. $^*p < 0.05$.

NPs on the nematode lifespan in Water Medium. As shown in Fig. 2, 2 ppb Pt NPs with an average diameter of 2.5 nm significantly prolonged the nematode lifespan, by 18%, as compared with the control worms ($p < 0.001$). However, the worms cultured in 10 and 50 ppb Pt NPs-Water Medium did not show any significant differences in their mean lifespans as compared with the control worms (Table 1). When the control worms survived much longer than those in normal trials, with a mean lifespan of more than 15 d instead of the usual lifespan of about 11 d, the lifespan-extending capability of Pt NPs was markedly weakened (Table 1, Trial 4). We speculate that this weakening of lifespan-extending capability was caused by the lower stresses in uncontrolled exotic environments such as oxidative stress, temperature stress, *etc*.

ROS accumulation in C. elegans

Next we examined the protective effect of Pt NPs on the ROS accumulation in *C. elegans* induced by paraquat. As shown in Fig. 3, 0.2 M paraquat-treated worms cultured in 5 ppb Pt NPs-Water Medium showed less ROS accumulation than those cultured in 0 ppb Pt NPs-Water Medium, significantly by 34%. The ROS accumulation in the worms treated with paraquat was significantly higher than that in the worms without paraquat treatment (Table 2), but no significant differences were found among the paraquat-free worm groups (Table 2). These findings indicate that the worms cultured in 5 ppb Pt NPs-Water Medium exhibited better

Table 2. Effects of Pt NPs on ROS Accumulation in *C. elegans* without Treatment with Paraquat

Paraquat	0 ppb +	0 ppb −	2 ppb −	5 ppb −	10 ppb −
RF	1	0.416	0.421	0.474	0.466
p	—	<0.05	<0.05	<0.05	<0.05

The effect of Pt NPs on ROS levels in nematodes without treatment with 0.2 M paraquat was investigated. RF, DCFH-DA relative fluorescence in *C. elegans* compared with the DCFH-DA fluorescence intensity of paraquat-treated worms cultured in 0 ppb Pt nps-Water Medium, which was normalized to 1. *p*, comparison with paraquat-treated worms cultured in 0 ppb Pt nps-Water Medium by the Welch test ($n = 10$–15).

tolerance of paraquat than the control worms. The survival ratio of the Pt NPs-treated worms after paraquat treatment was almost 10% higher than that of the control worms without Pt NPs treatment (data not shown), but the ROS-scavenging effects of Pt NPs decreased when the NPs concentration was 10 ppb (Fig. 3).

Discussion

ERW exhibits a low redox potential, from −200 to −800 mV,[1)] and antioxidant effects of it have consistently been reported.[1,3–10)] Based on previous studies and the free-radical theory of aging,[30)] we hypothesized that since ERW can alleviate ROS accumulation, it also has a positive effect on an animal's lifespan, but there is a concern that the antioxidant capability of ERW might not be sufficiently strong to be assayed accurately. We have examined the lifespan-extending effect of ERW on *C. elegans* using S medium, a traditional aqueous nematode growth medium consisting of multiple salts, but no significant differences were found.[12)] To increase the sensitivity of the evaluation system, we designed Water Medium, which contains only sample water, and used HEPES buffer to adjust the pH to 7.0. We found that ERW in Water Medium significantly extended the lifespan of *C. elegans*. We believe that Water Medium is closer to the natural physiological conditions for nematodes than S medium.[12)] In this study, we continued to use Water Medium to investigate the mechanism of the lifespan-extending effect of ERW.

TI-200 ERW prepared by electrolysis of 2 mM NaOH solution, is considered the simplest research model for ERW to date. Theoretically, it consists only of NaOH, H_2, contaminating trace metal ions, and nanoparticles like Pt NPs. Since the control solution for ERW contains an equal amount of NaOH, we hypothesize that its lifespan-extending capability is due to the hydrogen molecules dissolved in ERW, the trace amount of Pt NPs derived from the electrodes, or a combination of hydrogen and Pt NPs.

TI-200 ERW is known to contain high concentrations of molecular hydrogen, at about 0.6 mM, but the concentration decreases rapidly to less than 25 μM after pH adjustment.[12)] Although hydrogen molecules have been consistently reported to have potent antioxidant effects,[13–19)] the effects of molecular hydrogen at concentrations below 25 μM on nematodes remain to be investigated. In the present study, we evaluated the effect of dissolved hydrogen on the lifespan of *C. elegans*. Our findings that the worms cultured in a 80% H_2/20%O_2 atmosphere did not show a longer lifespan and that the worms cultured in pre-vacuumed ERW (to expel the dissolved hydrogen in ERW) survived significantly longer than the control worms suggest that the lifespan-extending effect of ERW is not due to the dissolved hydrogen in ERW.

On the other hand, it has been observed that during the electrolysis process a trace amount of Pt NPs with sizes of 1–6 nm become dissolved in ERW at concentrations of 0.1–2.5 ppb.[12)] Pt NPs are newly discovered antioxidants that have been reported to extend the lifespan of nematodes significantly. However, it has also been reported that concentrations higher or lower than 0.5 mM Pt NPs showed no significant effects on the nematode lifespan.[21)] These findings suggest that such low concentrations of Pt NPs in ERW might not be able significantly to affect the nematode lifespan in S medium, which happens to be in good accordance with ERW. Hence, we examined the lifespan-extending capability of Pt NPs in Water Medium. We found that treatment with Pt NPs at 2 or 5 ppb significantly extended the lifespan of *C. elegans* as compared with the control worms. The finding that Pt NPs at less than 10 ppb can extend the nematode lifespan suggests that the lifespan-extending effect of ERW might be caused by Pt NPs.

In a previous study, we found an effect of ERW on the ROS accumulation in nematodes induced by paraquat using ROS detection reagent DCFH-DA. The results showed that ERW significantly scavenged ROS in nematodes.[12)] If the Pt NPs in ERW are the reason for nematode lifespan extension and ERW extends the nematode lifespan at least partly by scavenging ROS in nematodes, it should also be the case that the ppb levels of synthetic Pt NPs can alleviate the oxidative stress induced by paraquat in *C. elegans*.

In this study, we used a modification of the method used in our previous study to evaluate ROS accumulation.[12)] The method used in our previous study was taken from Kim *et al.*,[21)] and is an accepted method of evaluating ROS accumulation in worms cultured in S medium.[21,31,32)]

Compared with the osmolality of S medium (360 mOSm/kg), Water Medium is extremely hypotonic (25 mOSm/kg). A low osmolality leads to a significantly shortened lifespan,[12)] and apparently, weaker tolerance to ROS. When worms cultured in Water Medium for 5 d were treated with 0.4 M paraquat for 5 h, the mortality of the worm population was 90%, and no significant improvements were found in the worms cultured in ERW as compared with the control worms (data not shown). Hence, in this study we reduced the treatment with paraquat from 0.4 to 0.2 M. Furthermore, we changed the washing buffer from M9 to a pH-neutralized control water sample, and changed the dye buffer from Hank's solution to a pH-neutralized control water sample. The results indicated that 2 and 5 ppb Pt NPs significantly alleviated oxidative stress in *C. elegans* and increased survival after paraquat treatment. These findings are in accord with our previous findings for ERW. The present data strongly suggest that trace amounts of Pt NPs are responsible for at least some of the therapeutic effects of ERW.

Compared to other antioxidants such as blueberry polyphenols, vitamin E, and tocotorienol that have been reported to extend the lifespan of *C. elegans*,[33–37)] the lifespan extending capability of ERW is considered to be extremely subtle, because ERW did not extend the lifespan of *C. elegans* in S medium, in which other antioxidants extended the lifespan. Kim *et al.* reported that lifespan extension due to synthesized Pt NPs was observed in S medium only when the concentration of Pt NPs was exactly 0.5 mM (100 ppm).[21)] Higher or lower concentrations of Pt NPs did not extend the lifespan. Because of the low lipophilicity of Pt NPs, it is not easy for Pt NPs to penetrate the cell membrane and enter the animal cells. Kim *et al.* conjugated Pt NPs with HIV-1 TAT, a cell-penetrating peptide, to increase the perme-

ability of the cell membrane, and found that TAT-Pt NPs extended the lifespan of *C. elegans* at 5 μM (1 ppm), a 100-fold smaller concentration than non-TAT-coated Pt NPs. Meanwhile, the lifespan extension capability fell when the concentration of TAT-Pt NPs was 50 μM, in spite of lowered ROS levels in the nematodes.[38] Only 1.8% of fluorescein-labeled TAT-coated Pt NPs was internalized into the nematodes. These results indicate that Pt NPs scavenges ROS, and extends animal lifespan in a very subtle way, in accord with our results that Pt NPs did not extend the lifespan of *C. elegans* in a dose-dependent manner.

With reference to the researches of Kim *et al.*,[38] the synthesized Pt NPs internalized into nematodes might have been less than 1 ppb in our research. The recent epigenetic oxidative redox shift theory of aging suggests that moderate oxidative stress extends the lifespans of animals, while both excess and insufficient oxidative stress result in shortening of lifespan *via* mitochondrial dysfunction.[11] Further detailed investigation is needed to determine the mechanism of Pt NPs in ERW on ageing. The complication of ROS theory causes a dilemma when people want to improve their health by using antioxidants. Since the amount of Pt NPs in ERW is so small, it is highly likely to have no side effect in daily drinking, which may explain why ERW is safe to drink.[2,39]

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