

Simulating the Effect of Liquid CO₂ on *Cryptosporidium parvum* Oocysts in Aquifer Material

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Abstract: The effects of liquid CO₂ injection on the viability of *Cryptosporidium parvum* oocysts were evaluated. A laboratory study was designed to test the effects of saturated CO₂, freeze-thaw cycles and different freezing protocols on *C. parvum* oocysts in aquifer material. Oocysts were exposed to a saturated solution of CO₂ at room temperature for 1-, 4-, 8-, and 12-h intervals and their viability was compared with controls. One- and three-cycle freeze-thaw experiments on oocyst survival were conducted. Inactivation of oocysts was assessed for: (1) rapid freezing and rapid thawing and (2) gradual freezing and rapid thawing. Exposure to 1 atm of CO₂ in water at room temperature had a negligible effect on oocyst viability. Average oocyst viability after the one- and three-cycle freeze-thaw experiments was 24.7 and 2.7%, respectively. The average oocyst viability associated with the rapid freeze-thaw and gradual freeze-thaw experiments was 11.3 and 26.2%, respectively. Freezing associated with injection of liquid CO₂ into aquifers would be the factor inactivating oocysts; to cause a 3-log decrease in oocyst viability multiple injections may be required.

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Introduction

One of the greatest concerns facing the drinking water industry today is contamination of public water supplies with *Cryptosporidium parvum* oocysts because they are not inactivated by standard methods of disinfection. Studies have demonstrated that both surface waters (Le Chevallier et al. 1991) and groundwater (Moulton-Hancock et al. 2000) are susceptible to *C. parvum* oocyst contamination. The possibility of contamination by this protozoan pathogen must be taken into consideration by water utilities that need to equalize seasonal water demands by storing treated surface water in suitable aquifers that are extracted, re-disinfected, and distributed during peak demands.

If a well is used for injection and recovery, pathogens that accumulate in the aquifer near the well head may be recovered in higher concentrations in the first recovered volumes of water. This issue is of particular concern for organisms resistant to traditional disinfection techniques such as *C. parvum*. Thus, additional treatment would be required to protect the groundwater from contaminant pathogens.

In situ methods for disinfection may be needed to provide additional protection of the groundwater and enhance the quality

of the recovered water. One technique used for the remediation of well heads of decreasing yield and clogged by biological growth is the injection of inert liquid carbon dioxide that has been patented (Jaworowsky, "Method and apparatus water flow stimulation in a well," U.S. Patent No. 4,534,413 (1985); Catania and Catania, "Method of stimulation of liquid flow in a well," U.S. Patent No. 5,394,942 (1995)]. When liquid carbon dioxide contacts groundwater, rapid vapor expansion is created, carbonic acid is formed, and the pH of the water is lowered. In the process, the expanding vapor freezes the aquifer material in the vicinity of the well. The lowered pH combined with the pressurization results in the reestablishment of well production and removal of biogenic iron and calcium deposits. If the carbon dioxide pressurization process results in the inactivation of *C. parvum*, quantification of those effects would be useful to the utility using that well as a source.

To simulate the method of injecting critical-point CO₂ in a well head under controlled laboratory conditions two experiments were designed. The first was to determine if water saturated with CO₂ alone affected survival of oocysts in aquifer material at room temperature. The second set of experiments were designed to determine the effects of freeze-thaw cycles on oocysts in aquifer material under CO₂ saturated conditions, and the effects of rapid freeze-rapid thaw, and slow freeze-rapid thaw on oocyst survival.

Materials and Methods

Oocyst Purification

Feces from infected 6- to 14-day-old Holstein calves were processed using continuous-flow differential density flotation, as previously described (Jenkins et al. 1997). Less than 4 weeks transpired between oocyst purification and their use in experiments.

Aquifer Material Characterization

The aquifer material used in the experiments consisted of Pioneer Blastsand 4 and was a commercial sand that was processed in

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Table 1. Chemical Characteristics of Sand Used in Experiments

Characteristics	Parts per million
Nitrate	1.0
Phosphorus	17.0
Potassium	14.0
Calcium	5843.0
Magnesium	35.0
Zinc	0.2
Iron	2.7
Manganese	0.6
Copper	0.1
Sodium	55.0
Sulfur	6.0
Boron	0.2
Salinity	104.0 ^a
pH	8.9 ^b

^aConductivity of working solution equilibrated with 1 g material.

^bpH of 100 mL solution equilibrated with 1 g material.

Texas for sand blasting procedures. It was classified as a coarse type sand. This aquifer material was analyzed by the Texas A&M Soil Testing Laboratory in College Station, Tex. (Table 1).

Sentinel Chamber

Sentinel chambers have been described [Jenkins et al. 1999; "Method and apparatus for the use of sentinel microorganisms," U.S. Patent No. 6,146,823 (2000)]. They consisted of the basket of a microfiltration system (2.5 cm long, with an internal diameter of 0.7 cm; Osmonics, Livermore, Calif.) with a nylon 0.45 μm pore-size filter encased in one end; at the other (top end) a perforated cap secured a 60 μm pore-size mesh filter (Spectra/Mesh; Markson, Inc., Hillsboro, Ore). The design and dimensions of the sentinel chamber enable the contents of the chamber to equilibrate rapidly with the exterior environment, and at the same time contain the sentinel oocysts for easy recovery. The chambers contained approximately 0.8 g of dry sand, and were hydrated and inoculated with 2–5 million viable oocysts as previously described (Jenkins et al. 1999).

Apparatus Design

The experimental apparatus as described by Keller (1998) was comprised of a carbon dioxide bottle, high and low pressure regulators, rotometer, fine bubble diffuser, pressurized mixing chamber, and a manifold of three Lexan bowls with drains that contained the aquifer material and sentinel chambers. Gaseous carbon dioxide was supplied to the rotometer at 0.9 MPa and at approximately 46 $\text{cm}^3 \text{h}^{-1}$. In turn, the rotometer supplied carbon dioxide gas through a fine bubble diffuser to the pressurized mixing chamber that held 5.7 L of de-ionized water. As a result, the vessel pressure was manually maintained between 0.03 and 0.06 MPa by a pressure relief valve located on top of the vessel. At a feed rate of 46 $\text{cm}^3 \text{h}^{-1}$, the solution in the pressurized mixing chamber reached complete saturation after 10 min (Compressed Gas Association 1984).

A low-pressure regulator supplied the CO_2 saturated solution to the manifold of three replicate aquifer chambers. Each of the aquifer chambers was approximately three quarters filled with dry sand. Inside each of the aquifer chambers, a sentinel chamber containing sand inoculated with oocysts (as described above) was

connected to a brass fitting at the 0.45 μm filter end and secured by valve grease. To equalize the flow between the three chambers low-pressure regulators were located on top of each aquifer chamber. Tygon tubing conveyed the discharge from the aquifer chambers and was captured in 2 L Erlenmeyer flasks. The flow rate through the sentinel chambers inside the aquifer chambers was approximately $2.22 \times 10^{-6} \text{ m}^3 \text{ s}^{-1}$.

To simulate freeze/thaw cycles, the three aquifer chambers were immersed in crushed dry ice that was contained in a stainless steel pan. The stainless steel pan was insulated on all sides with fiberglass pipe insulation (Easy Heat, New Carlisle, Ind.). Additional strips of fiberglass pipe insulation were placed along the top of the pan and secured with metal clamps.

Oocyst Extraction from Aquifer Material

Oocysts were extracted from the aquifer material contained within the sentinel chamber as described by Jenkins et al. (1999).

Dye Permeability Assay

Details of this assay have been described (Jenkins et al. 1997). Further information concerning its applicability as an indicator of oocyst viability or potential animal infectivity when oocysts are subjected to environmental pressures has been discussed (Robertson et al. 1998). In the context of ultraviolet (UV) and chemical disinfection the dye permeability assay may overestimate oocyst viability (Black et al. 1996; Clancy et al. 1998). Viable oocysts were the sum of 2-4'-diamidino-2-phenylindole-negative (DAPI⁻), propidium iodide-negative (PI⁻) oocysts, and DAPI⁺PI⁻ oocysts; DAPI⁺PI⁺ oocysts were considered inactivated, as well as oocyst shells empty of sporozoites.

In vitro Excystation Assay

The method of in vitro excystation has been described (Jenkins et al. 1997).

Determination of Oocyst Concentration

Ten μL of a 100 μL sample of oocysts that had been stained with fluorescent antibody (Hydrofluor-Combo, Strategic Diagnostic, Del.) as described above were pipetted into the well of a Neubauer-Levy-Hausser counting chamber (Hausser Scientific, Hoarsham, Pa.) and counted as described by Jenkins et al. (1997). The mean of six subsamples was considered the density of oocysts.

Microscopy

Duplicate 10 μL aliquots of oocysts from each extracted sample were pipetted on Noble Agar (Difco, Kansas, City, Mo.) coated microscope slides and each aliquot covered with a cover slip. The agar slides were prepared by spreading 1 mL of 1% Noble Agar (Difco) solution at 55 °C on each slide and allowing the agar to air dry and solidify. All samples were examined using a Zeiss LSM-210 in conventional differential interference contrast (DIC) and epifluorescence mode using a triple excitation/emission filter set (Catalog No. 61001, Chroma Technology Corp., Brattleboro Vt.) with excitation bands at 390–410, 485–510, and 555–585 nm and emission bands at 450–475, 510–550, and 595–660 nm. A separate UV filter (excitation bands 310–395 nm) was used for

DAPI fluorescence (Jenkins et al. 1997). A Zeiss 100X/1.3 Plan-Neofluor DIC objective combined with 10× eyepieces was used for all microscopy procedures except for enumerations which were performed with a Zeiss 40X/0.85 Plan-Neofluor DIC dry objective.

Experimental Design

CO₂ Experiments

Oocysts in triplicate sentinel chambers were exposed to CO₂-saturated water at room temperature for 1-, 4-, 8-, and 12-h intervals. Following each individual test, oocysts were extracted from the aquifer material inside the three replicate sentinel chambers, and assayed for viability with the dye permeability assay. The 8-h test was conducted twice in order to perform both the dye permeability and *in vitro* excystation assays. The excystation assay was used as a check on the dye permeability assay.

Freeze-Thaw Experiments

Triplicate sentinel chambers containing oocysts were exposed to carbon dioxide at one and three freeze/thaw cycles. The two separate experiments were performed using the aquifer simulation device as described above. The saturated solution of CO₂ was circulated through the aquifer and sentinel chambers as described above. When the solution no longer discharged from the aquifer chambers, the sentinel chambers were considered to have reached a frozen state. The sentinel chambers were frozen after 105 min of exposure to dry ice. After the aquifer chambers and sentinel chambers remained frozen for 2 h, the dry ice was removed to initiate thawing. During the 225 min of dry ice exposure, the temperature of the sentinel chamber was -51.7°C as measured with a thermocouple. The aquifer and sentinel chambers completely thawed after 4 h at room temperature. This process was repeated three times to obtain the three freeze-thaw cycles.

Following each individual test, oocysts were extracted from the aquifer material inside the three replicate sentinel chambers, and assayed for viability with the dye permeability assay.

For the rapid freeze rapid thaw experiment a liquid nitrogen slush was made by pouring liquid nitrogen into an insulated plastic container placed on a freeze dryer (Virtronics, VirTis Co., Gardiner, N.Y.). By pulling a vacuum on the plastic container, the liquid nitrogen became slush. The temperature of liquid nitrogen in a slush-type state was approximately -205°C . Duplicate sentinel chambers were filled with aquifer sand, hydrated with distilled water, and inoculated with 5 million oocysts. The sentinel chambers were submerged upright into the liquid nitrogen slush by using insulated, self-closing forceps. The sentinel chambers remained submerged for 1 min before removal. They returned to room temperature after approximately 20 min. Oocysts were extracted from the aquifer material and assayed for viability with the dye permeability assay.

For the gradual freeze rapid thaw experiment, the procedure consisted of freezing the sentinel chambers in a -20°C freezer (Gibson Refrigerator, Model No. GTN142WK3, Dublin, Ohio). Three replicate sentinel chambers were partially submerged in a plastic rectangular tray filled with 1 L of de-ionized water. The sentinel chambers were held vertically in a plastic holder that was placed inside the plastic tray. Each sentinel chamber was prepared with aquifer material and oocyst inoculum as previously described. To determine the freezing rate, a thermocouple (Digital K/J Thermometer, Fluke) was placed inside one of the sentinel

chambers. The thermocouple was secured to the plastic tray and outer refrigerator door with duct tape. Temperature readings were recorded in 15 and 30 min intervals.

After the sentinel chambers reached a steady state of freezing, they were removed from the holder and placed in a 4°C refrigerator (Gibson Refrigerator, Model No. GTN142WK3, Dublin, Ohio) for 5 min; then the sentinel chambers were placed in a 37°C incubator for approximately 11 min. The thermocouple remained in one of the sentinel chambers while in the incubator and measured temperature changes. Oocysts were extracted from the aquifer material and assayed for viability with the dye permeability assay.

Baseline Test

To establish results at time zero, a baseline test was conducted at the beginning of each of the experimental procedures. The tests were performed in triplicate by using sentinel chambers, as described. Each sentinel chamber was prepared as previously described. Immediately following the sentinel chamber preparation, oocysts were extracted from the aquifer material and assayed for viability with the dye permeability assay. Results were compared to oocysts suspended in distilled water at 4°C .

Data Analysis

Statistical analysis of data was performed with Minitab statistical software (Minitab Inc., State College, Pa.). A probability value of at least 0.05 was used to determine significant differences between means.

Results and Discussion

Carbon Dioxide Experiments at Room Temperature

Oocyst Density Determinations

Results of the oocyst density determinations indicated that the efficiency of oocyst recovery from the aquifer material ranged from 16 to 61%. The duration of exposure to the aquifer material at room temperature in CO₂ saturated water appeared not to be a factor since at sampling times 0, 1, 4, 8, and 12 h recoveries of 20 ± 0.02 , 16 ± 0.02 , 61 ± 0.03 , and $31 \pm 0.02\%$, respectively, were obtained. Given the initial concentration of oocysts, we assumed that the oocysts recovered from the aquifer material represented the total oocyst population that was assessed for viability.

Baseline Viability Determinations

Viability of the oocysts extracted from the aquifer material immediately after inoculation (time 0) was not different from control oocysts suspended in water and kept at 4°C . The initial act of inoculation, therefore, did not affect the viability status of the oocysts.

CO₂ Experiments at Room Temperature

Based on *t*-test analysis at $p=0.05$, no significant difference existed between the incubated samples and the distilled water controls (93.0 ± 0.0 , 85.5 ± 2.1 , 80.5 ± 3.5 , and $81.3 \pm 3.5\%$ for 1-, 4-, 8-, and 12-h incubations, respectively) for any sampling time. The effect of CO₂-saturated water on oocyst viability was insignificant. Although the pH of the saturated carbon dioxide solution was approximately 3.8 at room temperature, it did not adversely

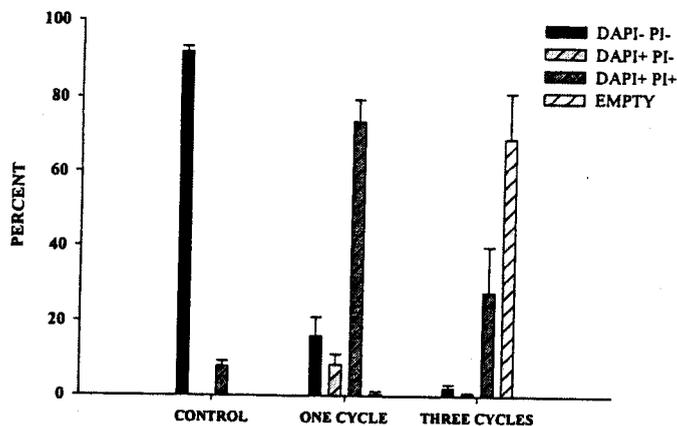


Fig. 1. Distribution of dye permeability categories of oocysts exposed to no freeze-thaw cycles, one freeze-thaw cycle, and three freeze-thaw cycles. Viable oocysts were sum of 2-4' diamidino-2-phenylindole-negative (DAPI⁻), propidium iodide-negative (PI⁻) oocysts, and DAPI⁺PI⁻ oocysts; DAPI⁺PI⁺ oocysts were considered inactivated, as well as oocyst shells empty of sporozoites. Error bars represent 1 standard deviation

affect oocysts. Campbell et al. (1992) reported that a 1 h exposure to 0.1 M HCl (~pH1) inactivated a significant fraction of suspended oocysts; but Hank's balanced salts solution acidified to pH 2.75 had no effect on oocyst inactivation. After 12 h of exposure to saturated solution of carbon dioxide, the *C. parvum* oocysts showed no significant difference in viability compared to the distilled water controls. Fayer and Leak (1984) bubbled an atmosphere of 50% CO₂ and air in an aqueous suspension of oocysts; their results, however, were inconsistent compared to controls. The results of the dye permeability assay for oocysts exposed to CO₂ for 8 h were not significantly different from the results of the in vitro excystation assay, and, therefore, not an overestimation of viability. Thus, exposure to 1 atm of CO₂ in water did not affect the viability status of the oocysts.

Freeze-Thaw Cycles

The temperature of carbon dioxide in the solid phase (dry ice) is -78.5°C. During the 225 min of dry ice exposure, a sentinel chamber temperature of -51.7°C was measured with a thermocouple. The phase change in the sentinel chambers exposed to dry ice occurred over a range of temperatures and not at a specific temperature. Because the temperature of the sentinel chamber was higher than the vitrification temperature of water, -143°C (Robards and Sleytr 1985), the system was not chemically immobilized. With a cooling rate of approximately 0.3°C min⁻¹ and an assumed nominal water permeability constant, it is probable that the internal water vapor pressure of the oocysts would equilibrate with the external water vapor pressure, and thus lose internal water (Mazur 1984).

A one cycle freeze-thaw test established a benchmark for additional test cycles. The single freeze-thaw cycle resulted in a percent average oocyst viability of 24.7±6.1 (Fig. 1). The control sample for this test had an average viability of 92%. The results of the three-cycle test showed a percent average viability of 2.7±1.2; the inactivated oocysts were observed as either DAPI⁺PI⁺ or empty (Fig. 1). Many of the empty oocysts appeared fragmented, mutilated, and not spherical or oval in shape. Jenkins et al. (1999) made similar observations of oocysts in soil that underwent several freeze-thaw cycles under field conditions.

The results of the freeze-thaw experiments demonstrated that the phase change from a liquid to a solid greatly impacted oocyst viability. The mechanical expansion and contraction of water between phases not only impacts the internal structure of the oocyst, but also acts as an external vice. As water becomes frozen, it expands and forms ice crystals both internal and external to the organism.

One possible interpretation of the one- and three-cycle freeze-thaw data is that each cycle inactivates a given fraction of the oocysts. If one cycle resulted in 24.67% (+/-6.15%) remaining viable oocysts, then two cycles would be predicted to have 6.08% remaining viable oocysts. Similarly, three cycles would be predicted to have 1.5% remaining viable oocysts. This compares well with the 2.67% (+/-1.21%) viable oocysts observed in the three-cycle test. Given the uncertainty in the fraction killed in the one-cycle test, the three-cycle test could have been as high as 2.93% remaining viable oocysts. Alternatively, it could have been as low as 0.635% remaining viable oocysts. The observed results for the three-cycle tests of 2.67% (+/-1.21%) remaining viable oocysts fall within the range of the predicted range of 0.635-2.93%.

If constant fractional removal can be further extended, then the number of freeze-thaw cycles required to reduce 5 million oocysts to less than one oocyst would take 11 cycles, and 12 cycles to inactivate all oocysts. To reach a 3-log decline in oocyst viability would require 8-9 freeze-thaw cycles. This prediction assumes all of the oocysts have the same statistical probability of destruction with no biological variability. Biological variability, however, may provide an inherent resistance to destruction by the freeze/thaw cycle. In a study in which sentinel chambers containing soil and oocysts were subjected to freeze cycles in surface soil under field conditions, Jenkins et al. (1999) reported 13 freeze-thaw cycles reduced the viability of the sentinel oocysts to less than 1% during the winter of 1997. During the winter of 1998, 3-5 freeze-thaw cycles reduced the viability of sentinel oocysts to approximately 25%. In both experiments, a significantly large proportion of empty oocysts were observed. The duration of exposure to freezing temperatures was not considered in these experiments, although Kato et al. (2002) reported that the percent inactivation of oocysts exposed to -10°C for 15 days in soil were not significantly different from oocysts in soil that were exposed to several freeze-thaw cycles. If freeze-thaw cycles are the principle source of oocyst inactivation, our results indicated that several freeze-thaw events would be necessary to inactivate *C. parvum* oocysts from recharged aquifers. Thus the use of liquid CO₂ as a method of eliminating oocysts from recharged aquifers may have practicable limitations.

Rate of Freeze-Thaw Tests

The results of an instantaneous freeze followed by a rapid thaw showed a percent average oocyst viability of 11.3±0.5 (Fig. 2). Cooling below the vitrification temperature has a significant impact on oocyst viability. The freeze rate of the gradual freeze-rapid thaw experiment was -0.01 to -0.1°C min⁻¹. The results of this test showed a percent average oocyst viability of 26.2±4.8, which was approximately two times greater than the rapid freeze-rapid thaw test. The gradual freeze-thaw test results were also approximately the same as one carbon dioxide freeze-thaw cycle. Although the gradual freeze-rapid thaw test was not as deleterious overall as the rapid freeze-rapid thaw test, significantly more empty oocysts were apparent compared to the rapid freeze test (Fig. 2).

The disparity between the two freezing temperatures appeared

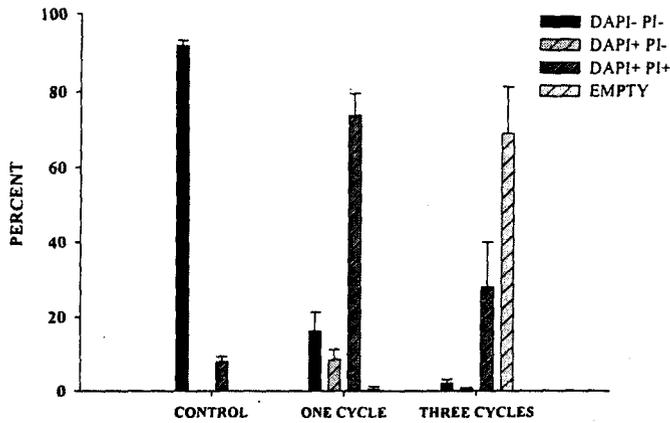


Fig. 2. Distribution of dye permeability categories of oocysts resulting from rapid freeze-rapid thaw and gradual freeze-rapid thaw treatments. Viable oocysts were sum of 2-4' diamidino-2-phenylindole-negative (DAPI⁻), propidium iodide-negative (PI⁻) oocysts, and DAPI⁺PI⁻ oocysts; DAPI⁺PI⁺ oocysts were considered inactivated, as well as oocyst shells empty of sporozoites. Error bars represent 1 standard deviation

to impact the viability results. The system of the gradual freeze test was not chemically immobilized because the temperature of the conventional freezer was higher than the vitrification temperature of water. The lower percentage of viable oocysts resulting from the rapid freeze/thaw test may be attributable to the different thermodynamic behavior of the liquid nitrogen slush. Robertson et al. (1992) observed the same disparity in inactivation when they subjected oocysts in reverse osmosis water to a "snap" freeze in liquid nitrogen, and to -22°C . The "snap" freezing resulted in complete inactivation of oocysts, whereas, at -22°C more than 30% remained viable after 21 h, and more than 1% remained viable after 775 h. Fayer and Nerad (1996) observed similar results: oocysts remained infective when exposed to -10°C for 7 days, but were inactivated by exposure to -70°C . Based on a *t*-test a significant difference existed between the means of empty oocysts for the gradual and rapid freeze tests at $P=0.01$. The test results demonstrated that the phase change from a liquid to a solid, as well as intracellular freezing and formation of ice crystals affected oocyst viability.

Conclusion

The effect of 1 atm of CO_2 in water at room temperature did not affect the viability status of the oocysts. The three-cycle freeze-thaw test as well as a single rapid freeze-thaw cycle reduced *C. parvum* oocyst viability but not to the 3-log decrease required. The results of this study suggest that injection of supercritical CO_2 into recharged aquifers to inactivate *C. parvum* may not be sufficiently effective since the supercritical temperature of -56.6°C is not below the temperature of vitrification, and multiple injections of supercritical CO_2 may be impractical. Because of the temporal and spatial limitations of this method of disinfection, the probability of its gaining regulatory credit is not large.

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