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CHEMICAL AND PHYSICAL FACTORS AFFECTING THE EXCYSTATION OF *CRYPTOSPORIDIUM PARVUM* OOCYSTS

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ABSTRACT: *Cryptosporidium parvum* oocysts were examined to ascertain excystation requirements and the effects of gamma irradiation. Oocysts and excysted sporozoites were examined for dye permeability and infectivity. Maximum excystation occurred when oocysts were pretreated with acid and incubated with bile salts, and potassium or sodium bicarbonate. Pretreatment with Hanks' balanced salt solution or NaCl lowered excystation; however, this effect was overcome with acid. Sodium ions were replaceable with potassium ions, and sodium bicarbonate was replaceable with sodium phosphate. Oocysts that received 200 krad irradiation excysted at the same rates as nonirradiated oocysts (95%), the excystation rates were lowered (50%) by 2,000 krad, and no excystation was observed by 5,000 krad. No differences were observed between the propidium iodide (PI) permeability of untreated oocysts and oocysts treated with 200 krad, while 92% of oocysts were PI positive after 2,000 krad. Most of the sporozoites exposed to 2,000 krad were not viable as indicated by the dye permeability assay. The oocysts irradiated with 200 and 2,000 krad infected cells, but no replication was observed. The results suggest that gamma-irradiated oocysts may still be capable of excystation and apparent infection; however, because the sporozoites could not reproduce they must not have been viable.

The identification of the mechanisms that trigger and carry out the process of excystation is critical for understanding how sporozoites are freed from oocysts prior to initiating the penetration of the hosts' intestinal cells. In vitro excystation is a parameter that has been used to measure the viability of sporozoites within oocysts (Campbell et al., 1992; Jenkins et al., 1997). Jenkins et al. (1997) reported that the in vitro excystation assay for potential infectivity of oocysts correlated with the in vivo mouse infectivity test and the in vitro dye permeability assay.

Several studies have examined the requirements for *Cryptosporidium parvum* oocyst excystation (Fayer and Leek, 1984; Reduker and Speer, 1985; Woodmansee, 1987; Current, 1990). Robertson et al. (1993) reviewed the excystation protocols developed by different research groups and recommended that, to achieve high excystation rates, *C. parvum* oocysts should be incubated with bile or bile salt for 4 hr at a temperature of 37 C, after a 1-hr treatment with acidified Hanks' balanced salt solution. Like other protozoan parasites (McKerrow et al., 1993), the excystation of *C. parvum* oocysts is possibly induced by proteolytic enzymes secreted by sporozoites (Nesterenko et al., 1995; Okhuysen et al., 1996; Forney et al., 1996, 1998) or by the structure of the oocyst wall or suture being changed by external chemical or physical factors. Oocysts that are killed by heat (60 C for 5 min) or formaldehyde (10% formalin) will not excyst (Jenkins et al., 1997), which suggests that viable sporozoites may be required for inducing excystation. However, most treatments that kill sporozoites also change the protein conformation of the oocyst wall and suture. Gamma irradiation is potentially capable of inactivating the contained sporozoites without affecting the structure of the oocyst wall and suture. If excystation requires proteolytic enzymes produced by either living or nonliving sporozoites, irradiation treatments that may block the production of the proteolytic enzymes or denature the proteolytic enzymes present in the cytosol of nonliving sporozoites should prevent in vitro excystation. Sincock et al. (1998) observed that the permeability of the oocyst wall to Sytox Green was changed by gamma irradiation levels between 100 and 900 krad. However, the effects of irradiation treatments on the permeability of the oocyst wall to propidium iodide (PI), which has been used as an indicator of oocyst inactivation or death (Campbell et al., 1992; Jenkins et al., 1997), have not been examined.

The objectives of the present study were to identify the minimum pretreatment and incubation medium requirements for oocyst excystation under in vitro conditions and examine the effects of sporozoite inactivation by gamma irradiation on excystation. A dye permeability assay (Anguish and Ghiorse, 1997) using PI was performed to determine whether the permeability of the oocyst wall to PI would be changed by exposure to gamma irradiation. In addition, a cell culture infectivity assay was performed to examine the effects of gamma irradiation on sporozoite viability.

MATERIALS AND METHODS

Oocysts

Cryptosporidium parvum oocysts were obtained from naturally infected 7–14-day-old calves in Tompkins County, New York. A sucrose/Percoll flotation method was used to extract oocysts from calf feces (Jenkins et al., 1997). After extraction, oocysts were stored at 4 C in water with antibiotics (100 U/ml of penicillin G sodium, 100 μg/ml of streptomycin sulfate, and 0.25 μg/ml of amphotericin B in suspension) and were used within 1 mo of collection.

Excystation procedure

A modification of the excystation method previously described by Campbell et al. (1992) and Jenkins et al. (1997) was used. Initially, 100-μl aliquots of a suspension of oocysts in a 1.5-ml microfuge tube were washed 3 times by centrifugation with 1 ml of Hanks' balanced salt solution (HBSS; Sigma, St. Louis, Missouri). In the routine procedure, approximately 7×10^4 oocysts were pretreated in a microfuge tube with 1 ml of 0.01 N HCl in HBSS (pH 2.3) for 1 hr on a heating block set for 37 C. After pretreatment, the oocysts were centrifuged at 13,000 *g* for 3 min. The supernatant was removed and discarded. The pelleted oocysts were resuspended in 10–20 μl of HBSS, and 2 μl of the oocyst suspension was placed in the center of a 12-mm-diameter circular coverglass that had been previously prepared by attaching a silicon gasket (13 mm in diameter and 0.381 ± 0.0762 mm thick, Swinnex 13; Millipore, Bedford, Massachusetts) to it with a thin layer of

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silicon grease. Then, 10 μl of liquid 1.5% ultra-low-gelling temperature agarose (Seaprep; FMC Bio Products, Rockland, Maine) was mixed with the oocyst suspension and allowed to solidify. The oocysts in the agarose matrix were washed 3 times at room temperature for 1 min each with 100 μl of HBSS that was applied to the agarose matrix in the well created by the silicon gasket. The agarose matrix was then washed for 1 min with 100 μl of excystation fluid (EF) (0.022 M sodium bicarbonate [$NaHCO_3$] and 0.0002 M sodium deoxycholate [$C_{24}H_{39}O_4Na$] in HBSS, pH 9.0). The silicone ring was filled with 32 μl of EF, and a glass microscope slide was lowered onto the ring without trapping air bubbles. The coverglasses were sealed to the slides using a melted mixture of equal portions of vaseline, lanolin, and paraffin. The sealed slides were inverted on racks and transferred to a 37 C incubator for 3 hr. After the incubation period, the slides were transferred immediately to a refrigerator where they were held at 4 C until the number of excysted oocysts was counted, usually in less than 6 hr. The slides were examined at a magnification of 400× on an inverted microscope (Zeiss IM) fitted with Hoffman modulation contrast optics. A total of at least 100 oocysts was counted. Oocysts containing sporozoites were considered unexcysted; oocysts containing no sporozoites were considered excysted. Excystation rates were calculated ([oocysts excysted/total oocysts counted] × 100). A minimum of duplicate samples were counted.

Variations in pretreatment and EF composition

A series of experiments was performed in which changes were made to the routine EF to examine the effects of the different components of the media on the excystation of oocysts.

Experiment 1: Oocysts in HBSS or water: Oocysts were held at 37 C for 3 hr in either water or HBSS without any added bile salts or bicarbonate and without any pretreatment.

Experiment 2: Replacement of HBSS with water: The HBSS in both the pretreatment fluid and in the EF was replaced with water, and the various combinations were examined for their effects on excystation.

Experiment 3: Examination of effects of major components of HBSS: Two of the major components in the formulation of HBSS are 0.8% sodium chloride and 0.1% glucose. The effects of these main components in the EF were tested. Oocysts were first treated for 1 hr with 0.1% aqueous glucose or 0.8% NaCl with and without added 0.01 N HCl before they were transferred to EF that had been made with either water or a 0.8% NaCl solution.

Experiment 4: Effects of acid on the suppressive effect of pretreatment with sodium chloride: A second pretreatment was applied to determine if the suppressive effect of HBSS on excystation could be overcome with additional acid treatment. The second pretreatments were for either 10 min or 1 hr with 0.01 N HCl. The EF in these experiments was made with water.

Experiment 5: Effects of other bile salts in the presence of sodium bicarbonate on excystation: To determine whether bile salts other than sodium deoxycholate would have similar effects on excystation, 2 additional bile salts, sodium taurocholate ($C_{26}H_{44}NO_7SNa$), and sodium taurodeoxycholate ($C_{26}H_{44}O_6SNa$), were used at equivalent molar concentrations in the preparation of EF in water. In these trials, pretreatment with either water or 0.01 N HCl was done for each of the bile salts examined.

Experiment 6: Replacement of sodium ions with potassium ions: In this experiment potassium deoxycholate ($C_{24}H_{39}O_4K$) was used; deoxycholic acid was dissolved in water, and an equivalent molarity of potassium hydroxide solution was added. The pH was adjusted to 7 with 0.1 N HCl. Sodium bicarbonate was replaced with potassium bicarbonate ($KHCO_3$) of the same molarity. The effects of the bile salt and the bicarbonate were examined after pretreatment with water at 37 C for 1 hr or with 0.01 N HCl in water at 37 C for 1 hr.

Experiment 7: Effects of pH on excystation: The addition of sodium bicarbonate to the EF increases the pH from 7.0 to 9.0. The effects of the pH changes on excystation were tested by varying the concentration of sodium hydroxide ions. After pretreatment with either water or 0.01 N HCl in water at 37 C for 1 hr, oocysts were transferred to 1 of 3 solutions: sodium deoxycholate at a pH of 7.0, sodium deoxycholate at a pH of 9.0, or the routine EF (water with sodium deoxycholate and sodium bicarbonate) at a pH of 9.0.

Experiment 8: Replacement of bicarbonate with sulfate or phosphate: To ascertain the importance of sodium bicarbonate, EFs were made containing the same molar concentrations of either sulfate (sodium sulfate, Na_2SO_4) or phosphate (sodium phosphate monobasic, NaH_2PO_4) at a pH of 9.0. Following pretreatment in water or 0.01 N HCl, oocysts were then transferred to 1 of these solutions for 3 hr at 37 C.

Experiment 9: Excystation in Tris buffer: Tris buffer (0.01 M, pH 7.2) was used instead of HBSS. For the pretreatment, the pH of 0.01 N HCl in Tris buffer was adjusted with HCl to 2.3. Excystation fluids were made with Tris buffer, and the pH was adjusted to 7.2 and 9.0 using NaOH.

Experiment 10: Effects of nickel and magnesium on excystation: Nickel sulfate ($NiSO_4$) and magnesium sulfate ($MgSO_4$) at the same molarity were tested with sodium deoxycholate. The pH was adjusted to 9.0 with HCl and sodium hydroxide.

Gamma-irradiation experiments

Gamma irradiation of oocysts was done using a cobalt 60 source in the Ward Laboratory of Cornell University (Ithaca, New York). *Ascaris suum* eggs that are known to be inhibited from developing by treatment with 200 krad irradiation (Reimers et al., 1986) were included as controls. The *A. suum* eggs were obtained from naturally infected swine feces and separated from the feces by sieving and repeated sedimentation in water at 4 C. Suspension of *C. parvum* oocysts and *A. suum* eggs in 1 ml of distilled water were placed in separate microfuge tubes and exposed to the cobalt 60 source for 1 hr (200 krad), 15.5 hr (2,000 krad), and 33 hr (5,000 krad). During the irradiation treatments, oocysts and eggs were placed on ice to avoid any increase in temperature. The temperature range during the irradiation was between 9.5 and 11.5 C. After irradiation, the oocysts were kept at 4 C for less than 6 hr.

Within 1 day after the irradiation treatments, the oocysts were excysted as previously described: 1 hr pretreatment at 37 C with either water or 0.01 N HCl and 3 hr in the EF made with HBSS at 37 C. The *A. suum* eggs were cultured in 0.5% formalin in a 28 C incubator for 2 wk after irradiation and examined microscopically for the effects of irradiation on the ability of larvae to develop within the eggshells.

Dye permeability assays

A modified dye permeability assay (Jenkins et al., 1997) was performed immediately after the irradiation treatment and 3 wk after the treatment. A stock solution of PI (Sigma) (1 mg/ml PI in 0.1 M phosphate-buffered saline) was added to aliquots of oocyst suspensions at a ratio of 1:10 (vol/vol), mixed gently, and incubated for 2 hr at 37 C in the dark. Samples were examined by epifluorescence and differential interference contrast (DIC) microscopy (Eclipse, E600, Nikon, Japan). An excitation band of 546/10 was used. At least 100 oocysts in each sample were examined.

Sporozoites membrane permeability examination

The dye permeability of the pellicle of the released sporozoites was examined using oocysts irradiated with 2,000 krad. Oocysts were pretreated with 0.01 N HCl in HBSS for 1 hr at 37 C. The oocysts were incubated in the EF for 10 min at 37 C, and then PI (1 mg/ml in 0.1 M phosphate-buffered saline [PBS]) was added to aliquots of the sample in microfuge tubes at a ratio of 1:10 (vol/vol). Immediately after the PI was added, the free sporozoites were observed by epifluorescence and differential interference contrast (DIC) microscopy (Eclipse E600). An excitation band of 546/10 nm was used. One-hundred sporozoites were identified and scored as either PI positive (nonviable) or negative (viable).

Cell culture infectivity examination

HCT-8 cells were used. In a microfuge tube, 10^5/ml oocysts were placed in 1 ml of 9 parts 1× PBS and 1 part 0.0525% sodium hypochlorite. The samples were vortexed and left to stand for 8 min. The samples were washed with 1× PBS twice using centrifugation. After the washing, 1 ml of prewarmed growth medium was added and vortexted for at least 1 min. The concentration of the oocysts in this stock sample solution was counted using a hemacytometer to verify at least 10^5 oocysts/ml. For the 10-fold dilution series, 5 sterile microcentrifuge tubes containing prewarmed 900 μl of growth medium were made. A 100-μl sample of the stock solution was added to the first tube to begin the series. From each dilution, a 150-μl sample was pipetted into labeled

TABLE I. Excystation in water or HBSS.*

Pretreatment solution (37 C for 1 hr)	Percentage excysted oocysts in water on HBSS (mean [SD])			
	Without sodium bicarbonate or sodium deoxycholate		With sodium bicarbonate and sodium deoxycholate	
	Water	HBSS	Water	HBSS
Water	0.25 (0.35)	5.96 (1.05)	39.46 (3.53)	73.50 (1.91)
0.01 N HCl in water	0.00 (0.00)	10.12 (1.09)	82.84 (5.39)	96.02 (2.56)
HBSS	0.17 (0.23)	6.13 (1.94)	8.88 (1.23)	9.31 (4.12)
0.01 N HCl in HBSS	0.33 (0.46)	9.32 (2.06)	52.51 (4.63)	96.87 (0.96)

* Excystation of oocysts of *Cryptosporidium parvum* in water or HBSS as the excystation fluid (EF) without any added bile salt or additional ions or in EF with sodium bicarbonate and sodium deoxycholate after pretreatment for 1 hr in either water, HBSS, or acidified water or HBSS. Control oocysts treated with 0.01 N HCl and excystation fluid containing sodium deoxycholate and sodium bicarbonate in HBSS showed excystation rates of 87.1% and 85.6% (mean 86.35%).

LabTech II chamber slides (Nalge Nunc International, Naperville, Illinois), and the slides were placed in a 5% CO_2 incubator for 48 hr at 37 C. After the incubation, 100% methanol was applied to the slides to fix each well for 10 min. The fixed slides were transferred to the blocking buffer containing 30 ml of 1× PBS, 3 ml of 0.02% Tween 20 (polyoxyethylenesorbitan monolaurate; Sigma), with 600 μl of goat serum, and placed in a refrigerator overnight. The slides were treated with a primary rat antibody to *Cryptosporidium* (Waterborne, New Orleans, Louisiana). A secondary antibody (fluorescein isothiocyanate-conjugated anti-rat immunoglobulin; Sigma) was added, and an epifluorescence DIC microscope (excitation of 450–490 nm with a 520-nm barrier filter) was used to observe sporozoites and other life stages. The focus detention method–most probable number (FDM-MPN) assay developed by Slifko, Friedman, Jakubowski, and Rose (1997) and Slifko, Friedman, Rose et al. (1997) was applied using the information collection rule general purpose MPN calculator (online: http://www.epa.gov/microbes, version 1.0, Risk reduction laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio, 1995).

RESULTS

Variations in pretreatment and EF composition

Experiments 1 and 2: Oocysts held in water at 37 C for 3 hr did not excyst even when pretreated at 37 C with 0.01 N HCl in water (Table I). When the oocysts were held at 37 C in HBSS for 3 hr, a small percentage did excyst (4.75–10.89%). When oocysts were held for 1 hr at 37 C in water and then transferred to water containing sodium bicarbonate and sodium deoxycholate, 40% (SD = 3.53) excysted. If oocysts were directly transferred from water at 37 C to HBSS with sodium bicarbonate and sodium deoxycholate, 73% (SD = 1.91) underwent excystation. If oocysts were pretreated at 37 C with HBSS before being transferred to either water or HBSS containing sodium bicarbonate and sodium deoxycholate, no more than 10% excysted. If the pretreatment water was acidified with 0.01 N HCl, transfer of the oocysts to water or HBSS with sodium bicarbonate and sodium deoxycholate induced 83% (SD = 5.39) and 96% (SD = 2.56) excystation, respectively.

Experiment 3: When oocysts were pretreated with 2 of the individual components of HBSS, 0.1% glucose or 0.8% NaCl, the effects of glucose pretreatment on excystation appeared to be the same as the effects of pretreatment with water. Using the EF made with water, 37.45% (SD = 1.95) of 0.1% glucose-pretreated oocysts excysted, and using the EF made with HBSS, 74.95% (SD = 3.01) oocyst excysted. Sodium chloride at 0.8%, however, appeared to have an effect similar to that of HBSS. When oocysts were pretreated with 0.8% NaCl, only 11–12% of the oocysts excysted both in the EF made with water and HBSS. The number of excysted oocysts increased when 0.01 N HCl was added to the glucose or the sodium chloride pretreatment solution, 80% (SD = 2.21) excysted in the EF made with water, and 95% (SD = 1.19) excysted in the EF made with HBSS.

Experiment 4: When oocysts were pretreated with 0.8% sodium chloride and then transferred to 0.01 N HCl at 37 C, the effects of the suppression due to the NaCl was removed in as little as 10 min (Table II). Water alone did not remove the suppression of excystation. Oocysts that were pretreated with

TABLE II. Ability of acidification to overcome depressive effects of sodium chloride.*

First pretreatment solution (37 C for 1 hr)	Percentage oocysts excysting in EF† following a second pretreatment at 37 C (mean [SD]); Second pretreatment solution‡					
	None	Water	HCl (10 min)	HCl (1 hr)	NaCl (10 min)	NaCl (1 hr)
Water	36.88 (3.17)	ND	84.60 (4.79)	93.31 (0.47)	18.95 (1.56)	20.71 (3.45)
0.01 N HCl in water	74.37 (4.25)	81.77 (3.03)	ND	ND	77.70 (3.68)	71.94 (2.66)
0.8% NaCl	15.27 (0.90)	14.18 (4.97)	75.01 (1.73)	73.14 (7.95)	ND	ND

* Excystation of oocysts of *Cryptosporidium parvum* after pretreatment for 1 hr at 37 C in water, acidified water, or 0.8% sodium chloride, followed by a second pretreatment with either water, acidified water, or 0.8% NaCl for either 10 min or 1 hr.
† EF made with water containing 0.022 M sodium bicarbonate and 0.0002 M sodium deoxycholate.
‡ HCl indicates 0.01 N HCl in water; NaCl indicates 0.7% NaCl.

TABLE III. Excystation with 3 different bile salts.*

Pretreatment solution (37 C for 1 hr)	Bile salt	Percentage excysted oocysts in water with added bile salt and/or sodium bicarbonate (mean [SD]) With or without sodium bicarbonate: Without $NaHCO_3$	With $NAHCO_3$
Water	None	ND	11.06 (1.10)
	Sodium deoxycholate	6.10 (1.55)	43.93 (0.35)
	Sodium taurodeoxycholate	12.89 (0.37)	74.50 (2.55)
	Sodium taurocholate	6.82 (1.68)	65.27 (1.09)
0.01 N HCl	Water	ND	14.02 (0.85)
	Sodium deoxycholate	4.71 (0.08)	91.66 (1.36)
	Sodium taurodeoxycholate	11.18 (1.62)	96.84 (0.37)
	Sodium taurocholate	5.36 (0.48)	88.69 (2.40)

* Excystation of oocysts of *Cryptosporidium parvum* in EF made with equal concentrations of 3 different bile salts, sodium deoxycholate, sodium taurodeoxycholate, or sodium taurocholate with or without the addition of sodium bicarbonate.

TABLE IV. Replacing sodium ions with potassium ions.*

Pretreatment solution (37 C for 1 hr)	Sodium or potassium ions	Percentage excysted oocysts in water with added bile salt and/or bicarbonate (mean [SD]) With deoxycholate or bicarbonate only, or with both: Deoxycholate only	Bicarbonate only	Deoxycholate and bicarbonate
Water	Sodium	2.85 (1.07)	6.17 (0.01)	39.71 (5.01)
	Potassium	6.73 (1.97)	4.56 (1.29)	37.09 (5.20)
0.01 N HCl	Sodium	5.35 (0.26)	10.43 (1.63)	84.16 (1.01)
	Potassium	11.06 (4.32)	6.83 (0.49)	83.08 (5.49)

* Excystation of oocysts of *Cryptosporidium parvum* with or without acid pretreatment in the EF made with equal concentrations of either sodium or potassium ions with the respective salt of deoxycholic acid, bicarbonate, or both. EF consisted of water with either 0.0002 M of the salt of deoxycholic acid; the carbonate was added at a concentration of 0.022 M.

0.01 N HCl in water for 1 hr at 37 C and then transferred to sodium chloride for periods up to 1 hr showed only minimal suppressive effects. When oocysts were pretreated with water and then transferred to sodium chloride, it appeared that some suppression of excystation occurred in as little as 10 min after the transfer to 0.8% NaCl.

Experiment 5: Neither sodium bicarbonate nor sodium deoxycholate alone was sufficient to cause a large percentage of oocysts to excyst whether pretreated with 0.01 N HCl or not (Table III). However, the addition of sodium bicarbonate alone caused slightly more excystation than the addition of only sodium deoxycholate. The 2 other bile salts examined, sodium taurodeoxycholate and sodium taurocholate, were as successful at inducing the excystation of oocysts as was sodium deoxycholate.

Experiment 6: Excystation was as high in the presence of only potassium ions as it was in the presence of sodium ions (Table IV). The excystation following pretreatment with water at 37 C resulted in 40% (SD = 5.01 and 5.20, for potassium and sodium ions, respectively) excystation in both sodium and potassium ions, whereas pretreatment with 0.01 N HCl resulted in 85% (SD = 1.01 and 5.49, for potassium and sodium ions, respectively) excystation.

Experiment 7: The adjustment of the pH of the EF with hydroxide ions did not allow excystation to take place. When pretreated with water and using EF with sodium deoxycholate at a pH of 7.0 or 9.0, excystation was 4.63% (SD = 2.07) and 4.72% (SD = 0.49), respectively. When the pH was 9.0, and both sodium deoxycholate and sodium bicarbonate were present in the EF, the excystation was 31.13% (SD = 0.87). When oocysts were pretreated with 0.01 N HCl for 1 hr at 37 C, the excystation in the presence of only sodium deoxycholate at either pH 7.0 or 9.0 was only 4.17% (SD = 0.87) or 5.14% (SD = 1.75), respectively. When oocysts were pretreated with 0.01 N HCl and both sodium deoxycholate and sodium bicarbonate were present in the EF, excystation was 71.49% (SD = 1.78).

Experiment 8: Following 1 hr in water at 37 C, 31.13% (SD = 1.24) of oocysts excysted in the EF of sodium deoxycholate and sodium bicarbonate, and 21.57% (SD = 1.53) of oocysts excysted when the sodium bicarbonate was replaced with sodium phosphate monobasic at a pH of 9.0. Sodium sulfate in combination with sodium deoxycholate also caused excystation but at reduced percentages compared to sodium bicarbonate or sodium phosphate. Sodium sulfate in combination with sodium deoxycholate caused 26.97% (SD = 1.24) excystation following water pretreatment and 35.02% (SD = 4.89) following acid pretreatment.

Experiment 9: If oocysts were held in EF made with Tris buffer at a pH of 7, the percentages of excysted oocysts were slightly higher than in EF made with water. The oocysts pretreated in water for 1 hr and transferred to the EF made with water showed 38.09% (SD = 1.90) excystation, whereas oocysts transferred to the EF made with Tris at pH 7.0 showed 60.91% (SD = 3.33) excystation. Following acid pretreatment in water, 90.90% (SD = 4.07) of oocysts in the EF made with water excysted, and 97.08% (SD = 1.15) of oocysts in the EF made with Tris excysted. Following acid pretreatment in Tris, 87.07% (SD = 0.93) of oocysts excysted in the EF made with water, and 96.95% (SD = 1.70) of oocysts excysted in the EF made with Tris. Pretreatment with Tris showed 21.78% (SD = 3.63) excystation in the EF made with water and 15.49% (SD = 2.79) in the EF made with Tris.

Experiment 10: The addition of nickel or magnesium ions, through the use of nickel sulfate and magnesium sulfate in the EF, reduced excystation. There was almost no excystation observed in the presence of the nickel ions (1.84% [SD = 0.33]) after pretreatment in water at 37 C or after acid pretreatment (0.96% [SD = 0.81]). The oocysts in the EF made with magnesium sulfate excysted only about half as well as those in the EF made with sodium sulfate. In the EF made with magnesium sulfate, 7.85% (SD = 0.04) of oocysts excysted after pretreatment in water and 19.43% (SD = 3.30) after pretreatment in 0.01 N HCl. The oocysts treated in the EF made with sodium sulfate showed 26.97% (SD = 1.24) excystation when pretreated with water and 35.02% (SD = 4.89) when pretreated with 0.01 N HCl.

TABLE V. Effects of gamma irradiation on oocyst excystation and permeability to fluorescent dye.*

Pretreatment solution (37 C for 1 hr)	Irradiation (krad)	Percentage excysted oocysts (mean [SD])		Percentage viable oocysts† (mean [SD])	
		Assayed immediately	Assayed after 3 wk	Assayed immediately	Assayed after 3 wk
Water	0	34.85 (9.07)	44.22 (5.03)	97.00 (0.71)	97.50 (0.71)
	200	26.62 (1.28)	32.72 (3.56)	91.45 (0.35)	76.00 (4.24)
	2,000	22.91 (2.66)	0.79 (0.18)	3.50 (2.12)	2.00 (0.71)
	5,000	1.51 (0.81)	ND	ND	ND
0.8% NaCl	0	ND	ND	98.25 (0.35)	ND
	200	ND	ND	91.60 (2.12)	ND
	2,000	ND	ND	3.75 (0.35)	ND
HBSS	0	ND	ND	99.00 (0.71)	ND
	200	ND	ND	87.60 (1.98)	ND
	2,000	ND	ND	3.75 (2.47)	ND
0.01 N HCl	0	95.93 (0.94)	96.85 (0.17)	79.85 (2.62)	93.80 (1.13)
	200	96.29 (0.49)	80.03 (4.18)	74.75 (10.25)	71.35 (2.33)
	2,000	49.67 (3.57)	0.32 (0.00)	2.00 (0.00)	1.75 (0.35)
	5,000	1.08 (1.06)	ND	ND	ND

* Oocysts of *C. parvum* received gamma irradiation at 0, 200, 2,000, or 5,000 krad. The oocysts were then excysted or stained with the fluorescent dye PI. After the gamma irradiation treatment, the oocysts were stored in water at 4 C for 3 wk before the excystation and dye permeability assay were performed.
† Percent viable oocysts = [PI^-/total oocysts] × 100.

Gamma irradiation

Excystation occurred at all levels of irradiation except at the highest dose (5,000 krad) used (Table V). The eggs of *A. suum* failed to develop after receiving 200 krad of gamma irradiation, whereas oocysts that were given 200 krad of irradiation, pretreated in 0.01 N HCl, and transferred to the complete EF underwent 96% excystation, equivalent to that of nonirradiated control oocysts. Oocysts treated with 2,000 krad of irradiation still excysted but at rates of only 50%. When oocysts received 5,000 krad of gamma irradiation, only 1% of the oocysts excysted.

If oocysts were irradiated and stored at 4 C for 3 wk before the excystation assays were performed, there was a reduction in the ability of the oocysts to excyst (Table V). Untreated control oocysts maintained 97.5% (SD = 0.71) excystation rates, but oocysts receiving 200 krad excysted at a rate of only 76.0% (SD = 4.24), rather than the 96% that occurred on the day that irradiation was completed. The oocysts that had received 2,000 krad of irradiation were apparently completely incapable of excystation 3 wk after having been treated.

TABLE VI. The result of cell culture infectivity assay, normalized MPN/ml positive oocysts.

Gamma irradiation	Normalized* cell culture MPN/ml positive oocysts	MPN/ml positive oocysts (95% confidence interval)
0 (control)	4.76×10^4	1.71×10^4–1.11×10^5
200 krad	2.41×10^2†	8.65×10^1–6.96×10^2
2,000 krad	2.16×10^2†	7.75×10^1–6.24×10^2

* MPNs are normalized by the hemacytometer ratio associated with the numbers of oocysts placed on the cell monolayer: hemocytometer ratios = sample hemocytometer counts/the highest hemocytometer counts. Raw MPN counts were then divided by the hemocytometer ratios.
† No clustering and no other life stages observed.

Dye permeability

The dye permeability of the 200-krad-irradiated oocysts was almost the same as that of the control oocysts (Table V). The oocysts irradiated at the 2,000-krad level showed only 2–3.8% viability using PI. Pretreatment with 0.01 N HCl slightly increased dye permeability (reduced the viability). The dye permeability of the oocysts 3 wk after irradiation treatment showed a decrease in oocyst viability. The control oocysts were still around 96% viable, the oocysts irradiated with 200 krad were between 70 and 76% viable, and the oocysts irradiated with 2,000 krad were mainly nonviable (PI^+).

Sporozoite membrane permeability examination

The membrane permeability of excysted sporozoites from the control (not treated) and 2,000-krad-irradiated oocysts was examined using PI, and the sporozoites that excysted following 2,000 krad of irradiation were dye permeable. For the untreated control oocysts, 85% of sporozoites were viable (PI^-), and they were observed to move immediately after emerging from the oocysts. In the case of the irradiated oocysts, no movement of sporozoites was observed, and 92% of the observed sporozoites that had excysted were nonviable (PI^+).

Cell culture infectivity assay

Results of the cell-culture infectivity assay (Table VI) indicated that oocysts gamma irradiated with 200 and 2,000 krad showed a 2-log reduction in the number of excysted oocysts. Few trophozoites were observed in cultures exposed to 200 or 2,000 krad, as well as no clustering of life forms of cells, indicating that replication was not occurring.

DISCUSSION

HBSS is widely used in the excystation of *C. parvum* oocysts (Campbell et al., 1992; Jenkins et al., 1997). The work here has

shown that oocysts will excyst in water containing sodium bicarbonate and sodium deoxycholate. Sporozoites, however, lyse because of the hypotonicity of the solution. On the other hand, sporozoites in HBSS containing sodium bicarbonate and sodium deoxycholate remain intact and are often observed to be motile. The replacement of HBSS with distilled water allowed the further examination of the effects of the different components of the EF on the excystation process.

In the present study, acid pretreatment (0.01 N HCl) was required for maximum excystation, although good excystation in HBSS, aqueous, or Tris buffer-based EFs occurred following pretreatment simply with water. On the other hand, the percentage of oocysts excysting after pretreatment with HBSS or 0.8% sodium chloride was decreased. If the HBSS or 0.8% NaCl was acidified with 0.01 N HCl, the percentages of excysted oocysts were similar to those that followed pretreatment with 0.01 N aqueous HCl. Thus, the depression in excystation caused by holding oocysts in HBSS was removed by the addition of acid to the HBSS. The suppressive effect caused by treatment with sodium chloride also could be overcome by treatment with 0.01 N HCl for only 10 min at 37 C. Fayer and Leek (1984) noted similar suppressive effects caused by saline and suggested that saline may prevent the excystation of oocysts and allow the sporozoites to survive until they reach the small intestines, where they can infect the host.

The importance of bicarbonate in the EF was verified by the fact that the adjustment of the pH of the EF with hydroxide ions did not allow excystation. However, CO_2 is probably not the important component added by bicarbonate. Oocysts kept in EF made with HBSS and sodium deoxycholate failed to excyst when inverted unsealed coverslips were maintained in a CO_2 incubator (data not shown). Also, sodium phosphate was as effective in inducing excystation as was sodium bicarbonate. The replacement of the sodium ions with potassium ions would indicate that sodium is not the essential component added by the sodium bicarbonate. However, phosphate was only poorly replaced by sulfate. Nickel and magnesium sulfate failed to induce excystation.

All the bile salts tested in this study (sodium deoxycholate, sodium taurodeoxycholate, and sodium taurocholate) were equally effective in inducing excystation. Patton and Brigman (1979) showed that sodium taurodeoxycholate resulted in higher excystation rates than other bile salts. Sundermann et al. (1987) tested the excystation of *Cryptosporidium baileyi* with bile salts from different species, and found that oocyst excystation of *C. baileyi* was not highly related to the host specificity of this parasite.

Oocysts that received 200 krad of irradiation remained impermeable to PI and excysted at levels similar to untreated control oocysts. Three weeks after irradiation, there was only a slight change in the percentage of PI-positive oocysts, and the majority of oocysts still excysted. The excystation of the rodent coccidium, *Eimeria nieschulzi,* was lowered after 15 krad of irradiation (Conder and Duszynski, 1977). After 20 krad irradiation, oocysts of the poultry coccidium, *Eimeria tenella,* were able to excyst and infect cells normally; however, the development of sporozoites into schizonts was significantly reduced (Gilbert et al., 1998). In the present study, gamma irradiation at 200 krad did not affect excystation; however, the irradiated sporozoites may have had damaged DNA (Madigan et al., 2000) that might have hindered their ability to develop further after infecting cells. This hypothesis was confirmed by the result of the cell-culture infectivity assay. Neither clustering nor further life stages were observed by the microscopic observation, although there were still 2.41×10^2 oocysts that were infective by the cell culture infectivity assay.

The oocysts that received 2,000 krad gamma irradiation were almost all PI positive and had a reduced ability to excyst immediately after treatment. After 3 wk, these oocysts had completely lost the ability to excyst. The sporozoites in the irradiated oocysts that excysted were permeable to PI and displayed no movement after excystation. The free radicals induced in the oocysts by the ionizing water (Pizzarello and Witcofski, 1967) could affect the enzymes in the oocysts or cause their release from sporozoites. Although the sporozoites were made permeable to PI by the gamma irradiation and were considered noninfective or nonviable, the enzymes involved in excystation may still have functioned to cause excystation. Although 2.14×10^2 oocysts still contained infective sporozoites as determined by cell count, neither clustering nor further life stages were observed in the cell-culture infectivity assay.

As time after treatment progressed, the enzymes involved in excystation may have decreased in function, thus accounting for the lack 3 wk later. The dose of 5,000 krad irradiation is known to inactivate enzymes (Madigan et al., 2000), and this enzyme inactivation may explain why only 1.5% of the oocysts excysted immediately after irradiation at this exposure level.

Okhuysen et al. (1996) and Forney et al. (1996, 1997) have tried to show that excystation is mediated by enzymes through the inhibition of excystation via protease inhibitors. The methods used in these experiments were to permeabilize the oocyst walls, and these same methods may damage the sporozoites or the contained enzymes. Basically, there are 2 methods by which excystation might be induced. In the first method, enzymes stored within the oocyst or within the contained sporozoites may be released upon appropriate signals, and these enzymes cause changes in the oocyst wall that cause the opening of the suture. In the other method, oocysts open because physical changes in the suture cause it to open without the need for any enzymes. The present work has shown that enzymes may be important for excystation, but the results are still inconclusive. It was thought that the irradiation experiments would show whether or not dead sporozoites could excyst. After 2,000 krad of irradiation, the PI-positive sporozoites appeared to be nonviable; nevertheless, they excysted quite successfully. The hypothesis that enzymes are involved in excystation is supported by the fact that oocysts that received 5,000 krad were incapable of excysting. The problem here is that this high dose of ionizing irradiation could also have affected the suture and thus have prevented the opening of the oocyst.

In conclusion, oocysts of *C. parvum* at 37 C will excyst following acid pretreatment in a very simple EF consisting of water containing nothing more than a bile salt and sodium bicarbonate. The bicarbonate can be replaced with phosphate but not with sulfate, and the sodium ions can be replaced with potassium ions. It appears that the specific bile salt is not a critical limitation on the system. Gamma irradiation with 200 krad neither decreases the number of oocysts immediately excysting nor causes the contained sporozoites to become permeable to PI. However, 200 krad irradiation decreases the ability of the ex-

cysted oocyst to infect cells. Higher doses of ionizing irradiation may both reduce the ability of oocysts to excyst and render the contained sporozoites nonviable as determined by PI staining. The role or source of any enzymes required for excystation by *C. parvum* oocysts has not yet been determined. The use of the inverted slide chamber and a system of minimal components should allow the system to be dissected more completely and ultimately lead to the determination of the actual means by which excystation occurs.

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