STUDIES ON THE EXCYSTATION OF *GIARDIA*

by

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A THESIS

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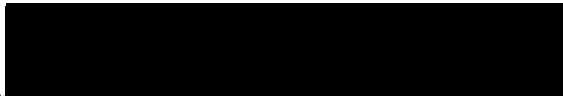

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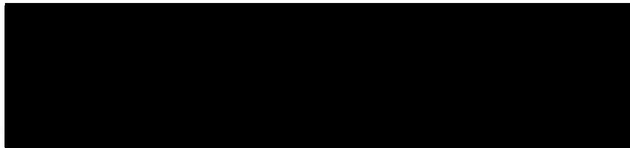

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INTRODUCTION

Life cycle and morphology. *Giardia* are protozoa belonging to the order Diplomonadida. They are parasites of the intestinal tract of a variety or vertebrates, including humans. The life cycle of organisms in this genus is composed of trophozoite and cyst stages. In the host, *Giardia* are found in the trophozoite form. Trophozoites are typically pyriform, 10 to 15 μm in length by 7 to 10 μm in breadth, possessing four pairs of flagella, a pair of characteristic median bodies, a central axoneme, and two nuclei. Trophozoites undergo active binary fission under favorable conditions. They may be found in large numbers *in vivo* in the upper small intestine attached by a ventral adhesive disc to the epithelial surface. Those which detach from the epithelium and pass through the remaining gastrointestinal tract are excreted either as trophozoites, which eventually disintegrate, or as cysts.

The mature *Giardia* cyst is typically ovoid, 7 to 12 μm in length by 3 to 6 μm in breadth, and consists of a tetranucleate organism enclosed in a rigid wall. Unlike the trophozoite, the cyst is the infective stage resistant to adverse environmental conditions and capable of surviving for weeks outside the host. When a cyst is ingested by an appropriate host, excystation occurs (the trophozoite emerges) in the upper small intestine and the life cycle is completed.

Pathogenesis. Although the majority of human infections are asymptomatic, colonization by *Giardia* (giardiasis) can result in a number of acute or chronic pathological conditions, notably intestinal discomfort and loose stools or diarrhea. *Giardia* infections have also been reported in association with malabsorption syndromes (1,12,35) and with liver and

bile tract disorders (54). The mechanism by which pathology occurs in giardiasis is still obscure. Mechanical obstruction, toxins, active competition with the host for nutrients, and synergism with other gut microorganisms have all been proposed as possible contributing factors (43,46,54).

Epidemiology. *Giardia* have a worldwide distribution; rates of human infection range from 1 to 30 percent depending upon the area (54). Although the incidence of this fecal-orally transmitted organism can be expected to vary inversely with hygienic standards, this parasite poses a public health problem even in areas where standards are presumed to be high. Numerous cases of giardial diarrhea are reported yearly in the United States among hikers and campers, and outbreaks of giardiasis have occurred in New York, Washington and New Hampshire, and among visitors to Leningrad, U.S.S.R. (3,6,7,8,13,47,60,68). Perhaps the largest outbreak occurred in 1954-55 when an estimated 50,000 cases of gastroenteritis were reported in Portland, Oregon. The only unusual finding within a group of randomly selected individuals was that of *Giardia* colonization at five times the normal incidence (66). Contaminated water was the implicated vehicle of transmission in all of the above outbreaks. Venereal transmission of *Giardia*, particularly among homosexuals, is becoming increasingly important epidemiologically (59).

History. *Giardia* was probably the first human intestinal protozoan observed (20). However, it did not come under careful scrutiny until the twentieth century. *Giardia* excystation was first observed in 1925 by Hegner (29) while examining cysts from the stool of an infected patient. Later, he described and made rough sketches of the excystation process

after observing cysts administered to and subsequently removed from rats (30,31,32). Two years later, Deschiens (17), in an attempt to determine if cysts survive gastric passage, reported seeing dead vegetative forms (trophozoites) following exposure of cysts to human "digestive juice" and to aqueous hydrochloric acid. In 1937, Armaghan (2) placed cyst suspensions directly into the stomach, duodenum, jejunum, ileum and caecum of rats and found that only those rats became infected which had cysts administered into the stomach or duodenum.

Although these workers did not determine the precise conditions necessary for *Giardia* excystation, their work suggested that temperature, moisture, gastrointestinal passage time, and certain digestive fluids might be involved.

STATEMENT OF PROBLEM

In the four decades since the work of Armaghan (2), no further contribution to an understanding of the excystation process of *Giardia* or of the conditions necessary for its induction has been reported. As recently as 1975 Lackie (42) wrote: "...attempts to bring about excystation of *Giardia lamblia in vitro* were without success...the factors which initiate it [excystation] do not appear to have been identified."

In view of this, the primary goals of this research are:

a) to induce *Giardia* excystation *in vitro*;
b) to define the conditions necessary for the induction of excystation;
c) to observe and describe the excystation process; and
d) to attempt to establish axenic cultures from excysted trophozoites.

MATERIALS AND METHODS

Cyst sources. Initial studies of excystation were performed on cysts obtained from dogs (University of Oregon Health Sciences Center [UOHSC] Animal Care Facility), monkeys (Oregon Regional Primate Research Center), and hospital inpatients (UOHSC Department of Clinical Pathology). Additionally, cyst-containing feces from animals were provided by other individuals and institutions. Later studies, and all those presented in this thesis, were performed using cysts obtained from one asymptomatic human male with giardiasis.

Cyst purification and storage. Cysts were purified by a modification of the procedures of Roberts-Thomson et al. (57) and Sheffield and Bjorvatn (61) as follows: Feces were suspended to a thin consistency in tap water and filtered successively through 1000, 710, 500, 250 and 177 μm nylon meshes (Tetko, Inc., Elmsford, N.Y.). Three to 5 ml of filtrate was placed on 3 ml of chilled 0.85 M sucrose in a 15 ml conical centrifuge tube, and the tube was centrifuged at 600g for 5 minutes at room temperature in a swinging-bucket rotor. When large volumes of feces were being purified, the sucrose gradient centrifugation was performed in 50 ml tubes; however, since higher purity was achieved with smaller tubes, 15 ml tubes were used whenever possible. The water-sucrose interface was removed, diluted 1:10 with water, and centrifuged at 600g for 5 minutes. The pellet was suspended in 3 ml of water and the sucrose gradient centrifugation repeated as many times as needed (usually once or twice) to achieve the desired purity. Following this the water-sucrose interface was again removed, diluted 1:10 with water, filtered under vacuum through a 20 μm nylon mesh (Tetko, Inc., Elmsford, N.Y.),

and centrifuged at 600g for 5 minutes. This pellet was resuspended in tap water to the desired cyst concentration. In all experiments except those in which the effect of storage temperature was studied, cyst suspensions were stored at 8 C. The purification procedure could be accomplished in an hour, yielding approximately 30 percent cyst recovery with purity acceptable for light microscopic examination and enumeration, and apparently undiminished cyst viability.

Growth medium. *Giardia* growth medium HSP-3, derived from Meyer's HSP-1 and HSP-2 media (45), was used in the excystation and cultivation of trophozoites. This medium has the following formulation: 85 ml Hanks' Phytone broth, 20 ml Seitz filter-sterilized heat-inactivated human serum, 7.5 ml NCTC-135 (Gibco, Grand Island, N.Y.), and 1.5 ml 1.0 M $NaHCO_3$; final pH 7.0. For culturing trophozoites the following antibiotics were added to HSP-3: 250,000 units potassium penicillin G, 0.02 g streptomycin sulfate, and 0.01 g gentamicin sulfate.

Excystation procedure. Unless otherwise stated, the following procedure was used in all excystation experiments: One volume of purified cyst preparation (usually 0.1 ml) was added to at least 10 volumes of excystation solution (saliva, gastric juice, HCl, etc.) and the mixture was incubated at 37 C for one hour. Following incubation, the suspension was centrifuged at 600g for 5 minutes at room temperature, and the pellet suspended in water. The suspension was centrifuged at 600g for 5 minutes and the pellet was suspended in HSP-3 (usually 0.5 ml) at 37 C. A depression-slide chamber was filled with the cyst suspension, sealed with a cover glass and paraffin-Vaseline, and incubated inverted at 37 C for one hour. The slide was then examined and the percentage of excyst-

ation determined as described in the subsequent section. Experiments showed that one hour of incubation in HSP-3 was sufficient to allow complete escape of those trophozoites capable of excysting.

Quantification of excystation and statistical analysis. Excystation was quantified by systematic examination of slide chambers at 300x on a Unitron model PH-BMIC inverted microscope. Eight fields were examined on each cover glass in the pattern shown in Figure 1. The fields were chosen such that they were midway between the outer edge and the center of the circular chamber, thus avoiding the sparsely populated outer edge and the densely populated center. This pattern was considered to be representative of the entire cover glass.

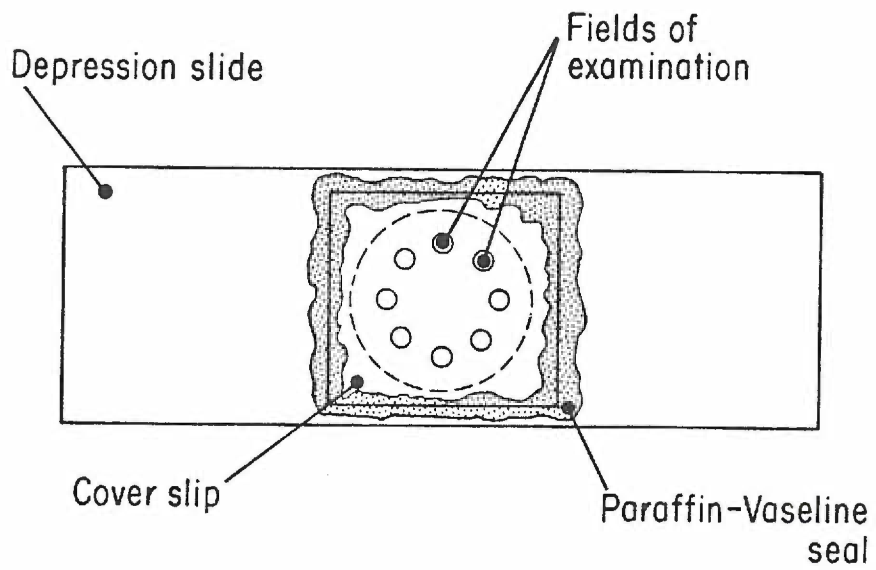
The percentage of excystation was determined by counting the number of intact cysts (IC), partially excysted trophozoites (PET), and totally excysted trophozoites (TET), and applying the following formula:

$$\% \text{ Excystation} = \frac{TET/2 + PET}{TET/2 + PET + IC} \times 100$$

In this formula, the number of totally excysted trophozoites is divided by 2 because every cyst in which total excystation occurred promptly yielded a pair of trophozoites. Totally excysted trophozoites, rather than empty cysts, were counted because a) empty cysts were sometimes difficult to detect due to their lack of refractility, b) excysted trophozoites attached rapidly to the glass and were easy to detect because of their active flagella, and c) the number of empty cysts present in a preparation due to causes other than excystation (e.g., death and trophozoite disintegration) was sometimes appreciable, particularly as

Figure 1

Pattern of the eight microscopic fields examined to determine percentages of excystation.



Depression slide
Fields of
examination
Cover slip
Paraffin-Vaseline
seal

the preparation aged. Subsequent trophozoite multiplication did not introduce counting errors since the generation time is approximately 18 hours (in M-5, a *Giardia* growth medium similar to HSP-3 [15]). Statistical significance was determined by Student's *t*-test; $P \leq 0.05$ was considered to be significant.

Photomicrography. Photographs were taken under phase contrast at 630x on a Zeiss Invertoscope D with Kodak SO-115 film.

Identification of inducing factor. In attempts to duplicate the conditions to which cysts are exposed *in vivo*, purified *Giardia* cysts were exposed to human saliva, gastric juice and duodenal-jejunal fluid obtained from normal individuals and hospital inpatients (UOHSC Division of Gastroenterology Diagnostic Unit). Fluid pH values were measured on a Leeds & Northrup model 7401 pH meter; the pH of one specimen of gastric juice was adjusted by the addition of 0.1 M HCl. Cysts were exposed at 37 C for two and four hours and then transferred into HSP-3. Due to a shortage of fluids, only one experiment was performed, and approximately 100 cysts were counted at each pH in each fluid.

A synthetic gastric fluid was prepared and used in attempts to identify the factor(s) in gastric juice inducing excystation. The synthetic gastric juice, a composite of those described by Hirschowitz (34) and Konturek (38), contained major components of normal human gastric juice in aqueous solution as follows: $NaHCO_3$ (25 mN), NaCl (40 mN), KCl (12 mN), $CaCl_2$ (12 mN), HCl (120 mN), and pepsin (1500 units/ml; Sigma Chemical Co., St. Louis, Mo.); the final pH was 1.6. Aliquots of the synthetic fluid were adjusted to different pH values with 1.0 M $NaHCO_3$ and excystation attempted in these solutions. Eight experiments

were performed and an average of 228 cysts was counted at each pH in each experiment.

Complete and component-varied synthetic gastric juices were prepared to identify factors other than pH which influence excystation. All solutions, except water and HSP-3 controls, were adjusted to pH 1.6. In eight experiments the solutions were compared for their ability to induce excystation. An average of 182 cysts was counted in each solution during each experiment.

To examine the influence of HCl on excystation, concentrated HCl was diluted with distilled water to the desired pH values. Eight excystation experiments were performed in each solution. An average of 196 cysts was counted at each pH in each experiment.

To determine whether hydrogen ion (H^+), chloride ion, or the combination of the two induced excystation, several inorganic acids were diluted in distilled water to a final pH of 2.0 and used to induce excystation. A control, consisting of water, was included in the experiments. Eleven experiments were performed, counting an average of 204 cysts in each acid during each experiment.

Time of acid exposure. To examine the influence of acid exposure time on excystation, cysts were exposed to HCl at various pH values for intervals from five minutes to four hours. Five experiments were performed, counting an average of 290 cysts at each pH-time combination in each experiment.

Temperature. The effect of cyst storage temperature on excystation was examined by the following procedure: Purified cysts were suspended in water and stored at either -13, 8, 21 or 37 C; the effect of the latter

three temperatures was studied in a single experiment using cysts purified from one fecal specimen. Cysts stored at -13 C were frozen in 0.5 ml aliquots by cooling to 8 C followed by transfer to -13 C; aliquots were thawed at 37 C as needed. At other temperatures, cysts were stored in 100 ml volumes from which aliquots were removed. Cyst viability was assessed periodically by excystation at pH 2.0 by the procedure described previously. Cyst viability at -13 C was compared to that observed at 8 C. An average of 440 cysts was counted at each temperature on each day that tests were performed.

A separate experiment examining cyst survival over 24 hours at 37 C was also performed. The procedure was the same as that described above, except that excystation was monitored hourly. An 8 C control was included in the experiment. An average of 117 cysts was counted at each temperature, each hour.

The effects of the acid and post-acid medium incubation temperatures were examined together. Cysts were incubated at pH 2.0 at either 8, 21 or 37 C by the usual procedure. The cysts exposed at one temperature were then divided into three equal portions (a total of nine for the three temperatures), and one portion from each temperature suspended and incubated in HSP-3 at either 8, 21 or 37 C. These suspensions were observed after 4 and 18 hours of incubation. Five experiments were performed. An average of 379 cysts was counted at each temperature combination in each experiment.

Post-acid incubation medium. The effect of the composition of the post-acid incubation medium on excystation was investigated by incubating cysts at pH 2.0 and then suspending them in solutions of varying complexity and pH. Three excystation experiments were performed, counting

an average of 278 cysts in each solution during each experiment.

In a second experiment, after acid exposure cysts were transferred into either a) Hanks' Phytone, b) Hanks' Phytone with serum, or c) HSP-3; all three media were adjusted to pH 7.0. Three experiments were performed. An average of 402 cysts was counted in each medium during each experiment.

Maturation, daily variation and viability. To study cyst variability with aging, purified cyst suspensions were prepared from different fecal specimens from the same host, stored at 8 C, and excystation followed in each suspension over a period of weeks. Three excystation replicates were performed daily. In a representative suspension, an average of 451 cysts was counted each day.

Variation in sensitivity to physical environment. Cysts were examined to determine if sensitivity to the physical environment changes as they age. Purified cysts were stored for 47 days at 8 C and excysted periodically using pH 2.0 HCl at various exposure times up to 120 minutes. Three excystation replicates were performed each day counting an average of 822 cysts at each temperature.

Excystation as an indicator of cyst viability. To examine the reliability of excystation as a measure of cyst viability, excystation and dye-exclusion were compared. Cysts were placed at pH 2.0 for time periods ranging from 0 to 240 minutes, then suspended and incubated in either HSP-3 or 0.1% eosin. Percentages of excystation and dye-exclusion (unstained cysts/total cysts) were then determined. Three excystation and eosin experiments were performed at each time period, counting an average of 216 cysts by each method in each experiment.

<u>Axenic cultivation of trophozoites</u>. The following procedure was used for the establishment of axenic cultures from excysted trophozoites: An aliquot of a suspension of purified cysts, containing at least 50,000 cysts, was placed in a small test tube and excystation induced at pH 2.0. HSP-3, containing antibiotics as described previously, was added to the tube and on alternate days replaced with fresh HSP-3 with antibiotics. Approximately 2000 units of Mycostatin Powder (E. R. Squibb & Sons, Inc., Princeton, N.J.) was added if needed to suppress the growth of fungal contaminants. Antibiotics were discontinued when the culture appeared to be free of contaminating microorganisms. The culture was considered to be axenic when no contaminants were detected by Gram stain and growth on blood agar and in thioglycollate broth. The culture was considered to be "established" three months after axenic cultivation.

RESULTS

I. Induction and description of excystation.

Excystation was induced in a variety of solutions; the process usually followed the course described below.

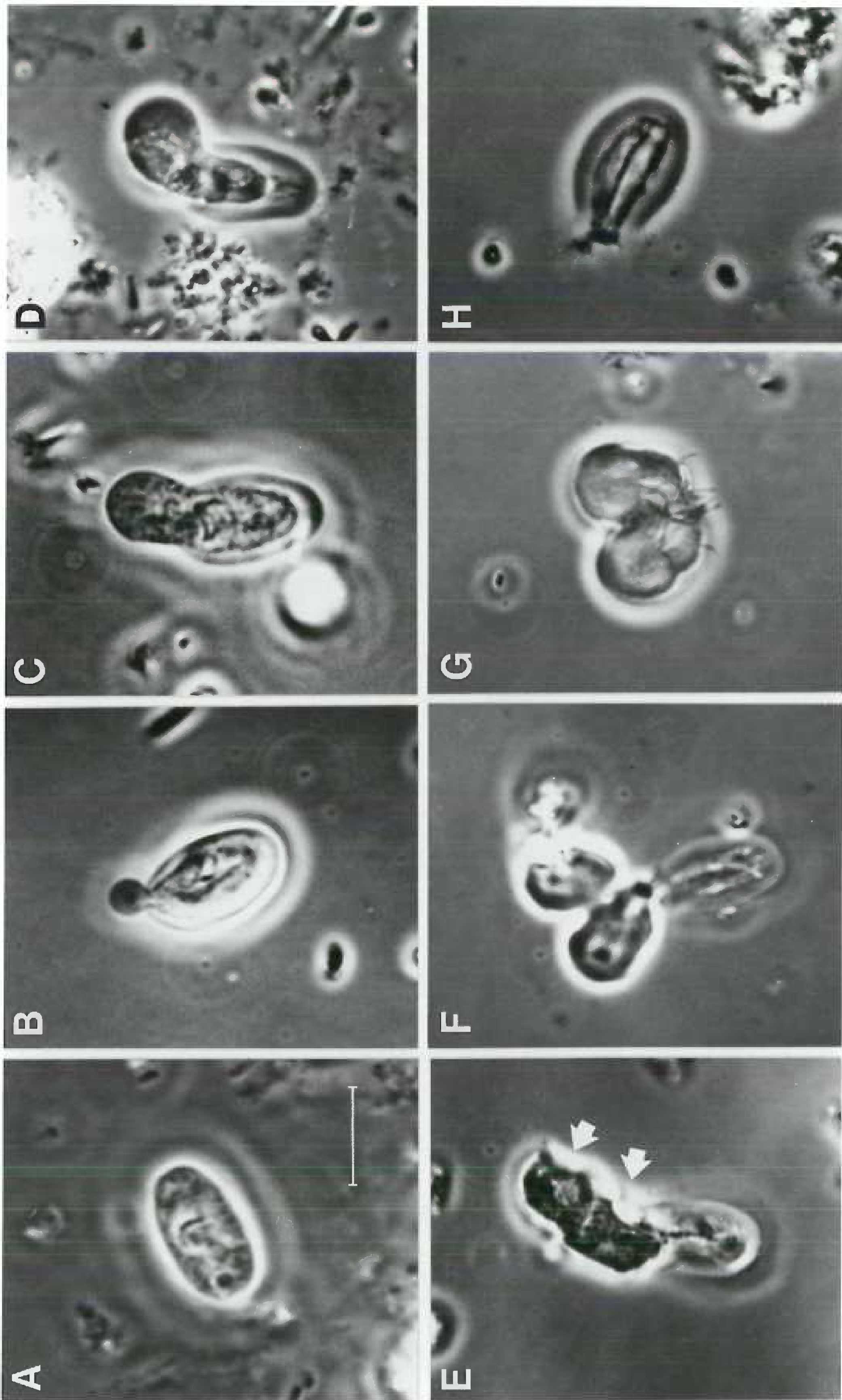
The initial stages of excystation began minutes after transferring cysts into HSP-3 (Figure 2). Initially, the trophozoite separated from the cyst wall, exhibited flexing movements, and then began to emerge from the cyst. The emergence resembled the extrusion of a fluid-filled balloon through a small hole, which was usually located in one end of the oval cyst. Active movement of flagella accompanied, and possibly aided, the escape of the trophozoite. Cell division always followed the emergence of a trophozoite from the cyst. Often differentiation and division were nearly completed before the trophozoite was free from the cyst wall. The entire process of excystation took place within 5 to 30 minutes following the transfer of cysts into HSP-3.

Abortive excystation was noted in three forms. In the first, the flexing movement inside the cyst ceased and the trophozoite expanded against the cyst wall. In observations of this type of cyst, no subsequent activity was seen over a period of several hours, suggesting loss of viability. In the second form of abortive excystation the trophozoites appeared to have died while attempting to escape from the cyst, resulting in a final stage which appeared like one of those in Figure 2B-D. In this stage, the cytoplasm of the intra- and extra-cystic portions of these partially excysted trophozoites appeared structurally normal. In the third form, cysts appeared similar to the one shown in Figure 2B, except that the protruding cytoplasm was devoid of any discernible

Figure 2

Representative steps in *Giardia* excystation. (A) Typical cyst; (B through F) sequential emergence of trophozoite(s) [arrows (E) indicate ventral adhesive discs of daughter trophozoites]; (G) excystation completed, division of daughter trophozoites continuing; (H) empty cyst.

Scale bar = 10 μm.



structures or organelles. In addition, the number and size of the protrusions varied; when multiple protrusions were present they were always localized in one area of the cyst surface. Protrusions apparently detached completely from some cysts and could be seen as refractile spheres in the medium. The frequency of cysts exhibiting abortive excystation seemed to increase with increasing cyst age.

Later experiments revealed that aberrant, as well as abortive, excystation could occur. Occasionally trophozoites attempted excystation through the side of the cyst (lateral excystation) rather than through one end (terminal excystation). In the former, excystation was almost always abortive. Unusual extra-cystic structures were rarely observed. These included motile trophozoite-like organisms approximately one-fourth to one-half normal trophozoite size, and rod-like or club-shaped structures resembling axonemes surrounded by cytoplasmic remnants, "wiggling" independently in the medium. The incidence of aberrant excystation and of unusual extra-cystic structures was greater in some fecal specimens than in others - even from the same host. In one human case followed from onset of symptoms through eight months of asymptomatic infection, most fecal specimens contained typical cysts yielding up to 60 percent excystation and normal trophozoites. Other fecal specimens contained cysts which appeared typical, but in which the ability to excyst was either poor or absent. Still other specimens yielded irregularly shaped cysts containing either normal or abnormal appearing trophozoites. These atypical cysts were likely to undergo aberrant and/or abortive excystation, and were found with increasing frequency in fecal specimens as the patient approached a three-month period of non-excretion.

II. Identification of inducing factor.

In experiments simulating *in vivo* conditions, it was found that (a) only those fluids with a pH of 5.0 or below induced excystation and (b) the percentage of excystation appeared to increase with decreasing pH (Table 1).

The influence of pH on excystation was examined further by inducing excystation in several pH-adjusted human digestive fluids. The results of this experiment are shown in Table 2. All fluids with pH values between 2.1 and 4.9 induced high levels of excystation; at higher pH values, little or no excystation was induced. In gastric juice the highest levels of excystation were induced between pH 2.1 and 4.0, with reduced excystation at pH 4.9 and none at higher pH values.

The results of inducing excystation in synthetic gastric juice (Figure 3) were similar to those found in experiments using human gastric juice. Peak percentages of excystation were observed between pH 2.0 and 3.2 with progressively diminishing levels above or below these pH values. Peak percentages of excystation were significantly greater than those at other pH values ($P < 0.05$).

A summary of the comparison of complete and component-varied synthetic gastric juices for their ability to induce excystation is presented in Table 3. With the exception of water and HSP-3 controls, all of the solutions induced virtually identical levels of excystation ($P > 0.05$); excystation in the control tubes was significantly lower than in the other solutions ($P < 0.05$). The only common factor in the excystation-inducing solutions was HCl in a concentration resulting in a pH of 1.6. The presence of salts and pepsin did not significantly alter the degree of excystation.

Table 1

Excystation of *Giardia* exposed to several human digestive fluids.

Fluid	pH	% excystation
Water (control)	6.8	0
HSP-3 (control)	6.8	0
Saliva	7.6	0
Gastric juice[a]	7.6	0
Gastric juice	5.0	10.9
Gastric juice[b]	2.4	40.8
Duodenal-jejunal fluid	5.7	0
Duodenal-jejunal fluid	4.3	10.6

[a] From gastric resection patient (patient 1).

[b] From patient 1, adjusted to pH 2.4.

Table 2

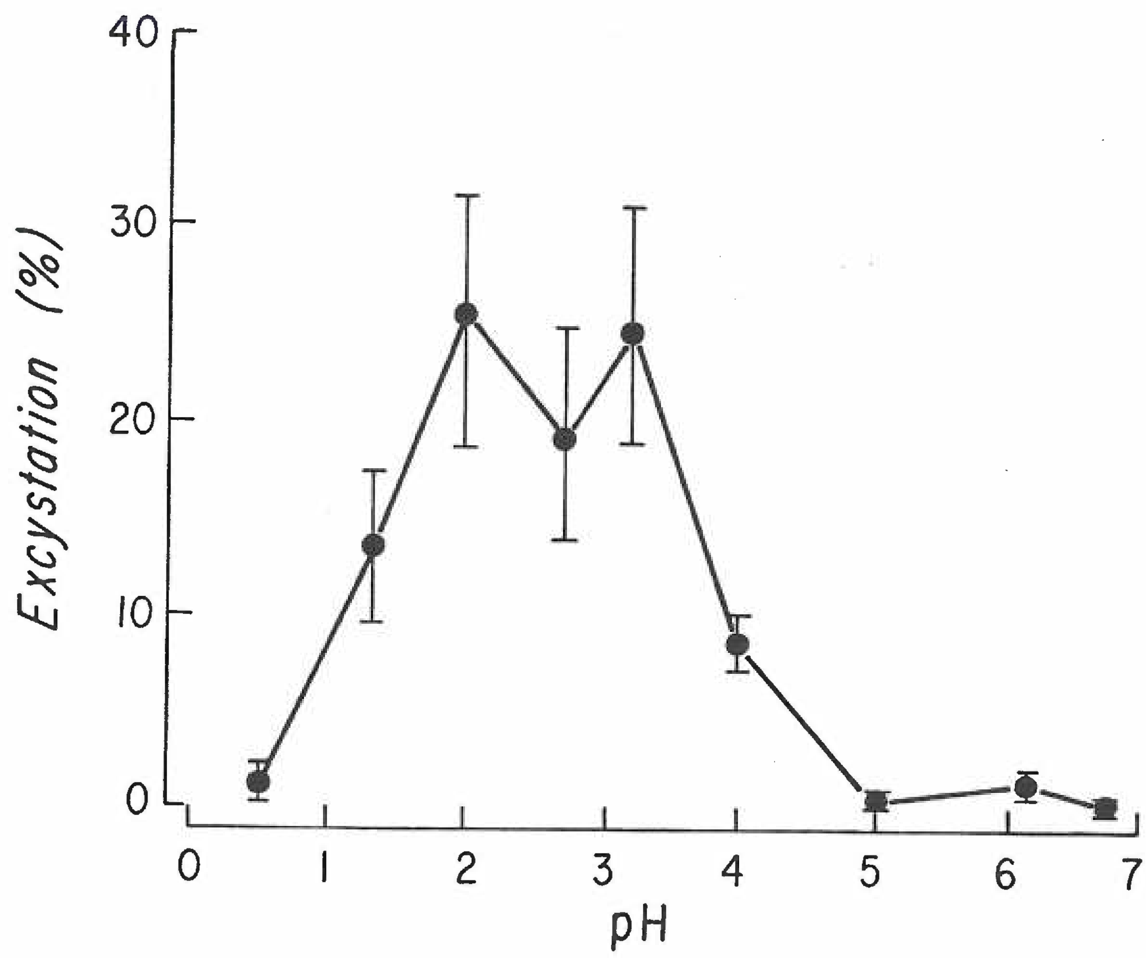
Excystation of *Giardia* exposed to several pH-adjusted human digestive fluids.

Fluid	pH	% excystation
Water (control)	6.8	0
HSP-3 (control)	6.8	0
Saliva	7.6	0
	2.3	43.8
Gastric juice	7.6	0
	6.8	0
	5.9	0
	4.9	18.0
	4.0	42.5
	2.9	30.3
	2.1	37.2
Duodenal-jejunal fluid	7.2	3.1
	2.3	48.6

Figure 3

Excystation of *Giardia* exposed to pH-varied synthetic gastric juice.

Vertical bars represent standard error of the mean.



Excystation (%)
40
30
20
10
0
0
1
2
3
4
5
6
7
pH

Table 3

Excystation of *Giardia* exposed to complete and component-varied synthetic gastric juice.

Solution	pH	Mean[a] % excystation ± S.E.M.
Complete (HCl + salts + pepsin)	1.6	25.8 ± 7.3
HCl + salts	1.6	23.0 ± 7.0
HCl only	1.6	23.2 ± 7.0
Water (control)	6.8	0.1 ± 0.1
HSP-3 (control)	6.8	0.0 ± 0.0

[a] Means are derived from an average of 1459 cysts counted in each solution.

The influence of HCl on excystation was examined; the results (Figure 4) revealed a pattern similar to those observed when pH-varied gastric juice (Table 2) and synthetic gastric juice (Figure 3) were used. The percentages of excystation at pH 1.3 to 2.7, although not significantly different from each other ($P > 0.05$) were significantly greater than those at other pH values ($P < 0.05$).

Exposing Giardia cysts to several inorganic acids at pH 2.0 revealed that each acid induced essentially identical levels of excystation ($P > 0.05$; Table 4).

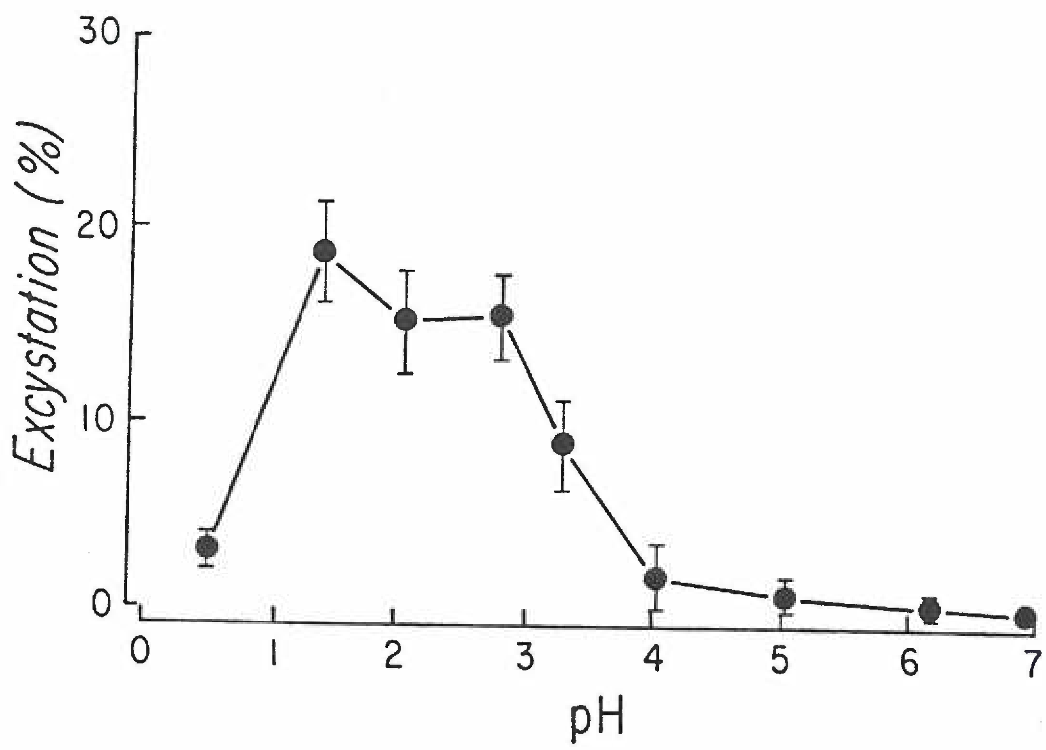
III. Effect of variation of physical environment.

A. Hydrogen and other ions. As shown previously, the induction and level of excystation is dependent upon pH (Tables 1 and 2, Figures 3 and 4); neutral or near-neutral pH solutions failed to induce significant levels of excystation regardless of their complexity (Tables 1 and 2, Figures 3 and 4). Except for H^+, the ions tested did not appear to influence the degree of excystation (Tables 3 and 4).

B. Time of acid exposure. The results of exposing cysts to HCl at various pH values for varying lengths of time are shown in Table 5. They indicate that a) the exposure time required for excystation is reduced as pH decreases, b) an optimum range of exposure times exists at pH values from 0.5 to 6.2, and at any one time, an optimum pH interval can be determined, c) the mean optimum exposure time at each pH value increases as the pH increases, and d) the mean optimum percent excystation at pH 0.5 and 2.0, although not significantly different from each other ($P > 0.05$), were significantly greater than those at 4.0 and 6.2

Figure 4

Excystation of *Giardia* exposed to pH-varied HCl. Vertical bars represent standard error of the mean.



30
20
10
0
Excystation (%)
0
1
2
3
4
5
6
7
pH

Table 4

Excystation of *Giardia* exposed to inorganic acids at pH 2.0.

Acid	Mean[a] % excystation ± S.E.M.
Water (control)	0.2 ± 0.2
HCl	17.4 ± 2.2
HNO_3	12.5 ± 3.0
$HClO_4$	15.5 ± 2.0
H_2SO_4	15.5 ± 2.6
H_3PO_4	19.4 ± 2.8

[a] Means are derived from an average of 2242 cysts counted in each solution.

Table 5

Excystation of *Giardia* at varying pH values and exposure times.

pH	Exposure time (minutes)										Mean optimum exposure time[c]	Mean optimum % excystation[d]
	5	10	15	20	30	60	90	120	180	240		
0.5	243[a]	256	231	--[b]	127	38	--	--	--	--	10	243
2.0	28	248	--	222	203	100	53	20	--	--	20	224
4.0	0	1	--	5	28	23	2	9	2	--	45	26
6.2	--	--	--	--	0	3	8	6	4	0	112	5

[a] Values represent percentage of excystation with reference to control tube (pH 2.0, 60 minutes) which is designated arbitrarily as 100%. Underlined values represent optimum percentages of excystation at a given pH which are not significantly different from each other ($P > 0.05$).

[b] Excystation was not determined.

[c] Average of exposure times at which optimum percentages of excystation are not significantly different from each other at a given pH ($P > 0.05$).

[d] Average of optimum percentages of excystation which are not significantly different from each other at a given pH ($P > 0.05$).

($\underline{P}$ < 0.05). In addition, the time interval during which optimum excystation occurs increases with increasing pH - 10, 20, 30 and 120 minutes at pH 0.5, 2.0, 4.0 and 6.2, respectively. When cyst exposure time is plotted versus $\log_{10}$ percent excystation (Figure 5), a linear relationship is seen at all pH values examined (r = -0.998, -0.983, -0.964 and -0.992 at pH 0.5, 2.0, 4.0 and 6.2, respectively). The slopes of these lines (k, which are equivalent to the excystation rate) vary directly with the H^+ concentration (r = -0.925; Figure 6).

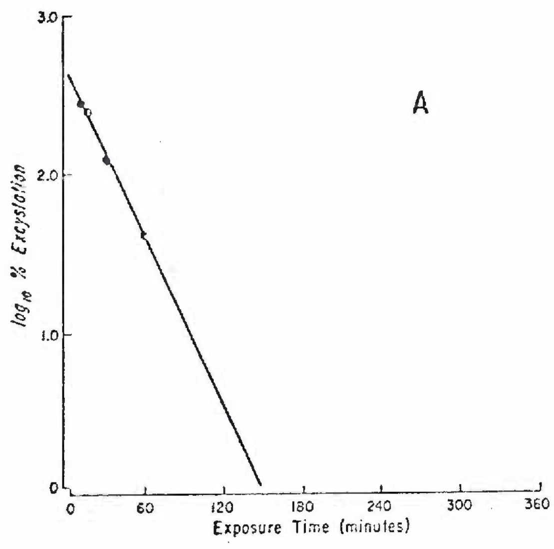
C. Temperature. The influence of temperature on cysts and excystation was examined by determining a) the effect of storage temperature, b) the effect of the temperature of the acid incubation solution, and c) the effect of the temperature of the post-acid incubation medium (HSP-3).

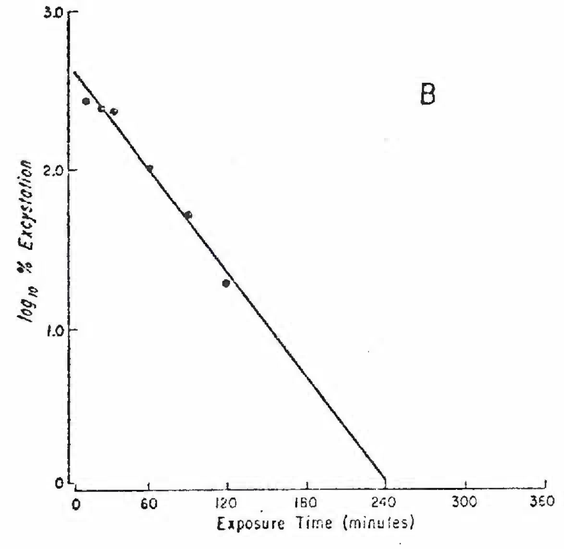
Representative results of the effect of storage temperature on cysts are summarized in Figures 7 through 11. Cysts stored at 8 C (Figure 7) exhibited higher levels of excystation for a longer duration than those stored at the other temperatures. The observed maximum percentage of excystation at each storage temperature decreased in the order 8 > 21 > 37 > -13 C. Viability at 8 C was observed through day 77 at which time the cyst suspension was depleted. At 21 C (Figure 8), cyst viability ranged from 5 to 24 days. Cysts stored at 37 C (Figure 9) lost viability within four days. Usually the percentage of excystation at this temperature was greatly reduced even after 24 hours, as indicated by an experiment in which excystation was monitored hourly. In this experiment (Figure 10), the percentage of excystation for cysts stored at 8 C remained at approximately 50 percent over the 24-hour period; at 37 C,

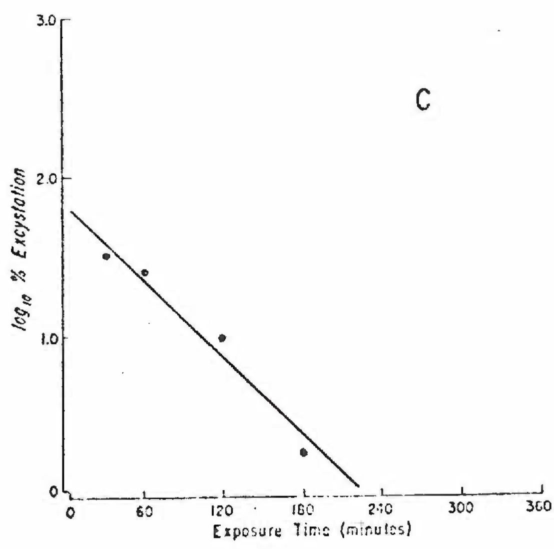
Figure 5

Rate of *Giardia* excystation at varying pH values and exposure times.

(A) pH 0.5, (B) pH 2.0, (C) pH 4.0, (D) pH 6.2 (see Table 5).


A
3.0
2.0
1.0
0
log10 % Excystation
0
60
120
180
240
300
360
Exposure Time (minutes)


B
3.0
2.0
1.0
0
log10 % Excystation
0
60
120
180
240
300
360
Exposure Time (minutes)


C
3.0
2.0
1.0
0
log10 % Excystation
0
60
120
180
240
300
360
Exposure Time (minutes)

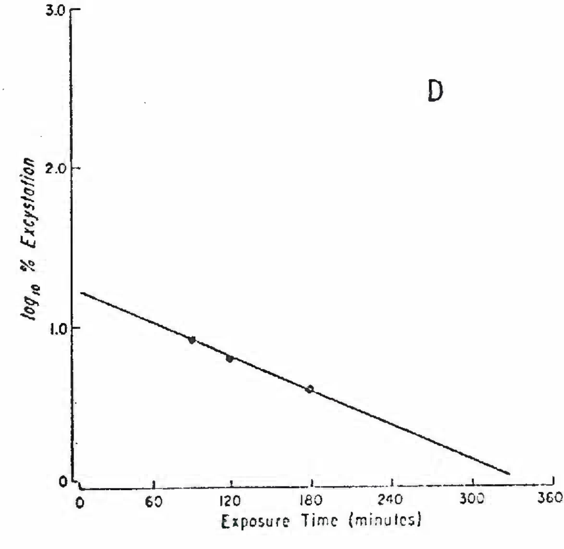

D
3.0
2.0
1.0
0
log10 % Excystation
0
60
120
180
240
300
360
Exposure Time (minutes)

Figure 6

Effect of pH on the rate (k) of *Giardia* excystation (see Figure 5 and Table 5).

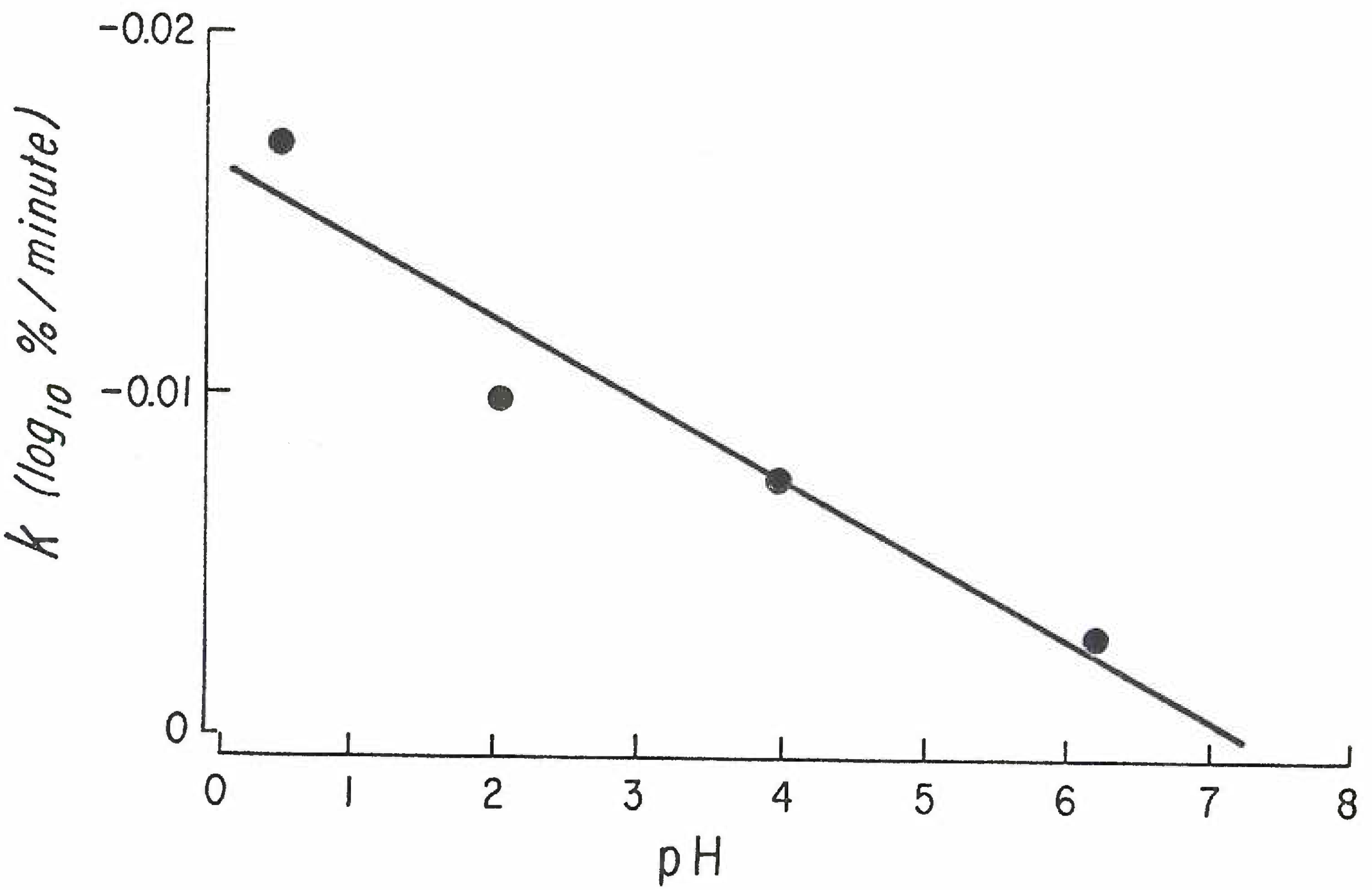

-0.02
-0.01
0
k (log10 % / minute)
0
1
2
3
4
5
6
7
8
pH

Figure 7

Effect of storage at 8 C on Giardia cyst viability as determined by excystation. Vertical bars represent standard error of the mean.

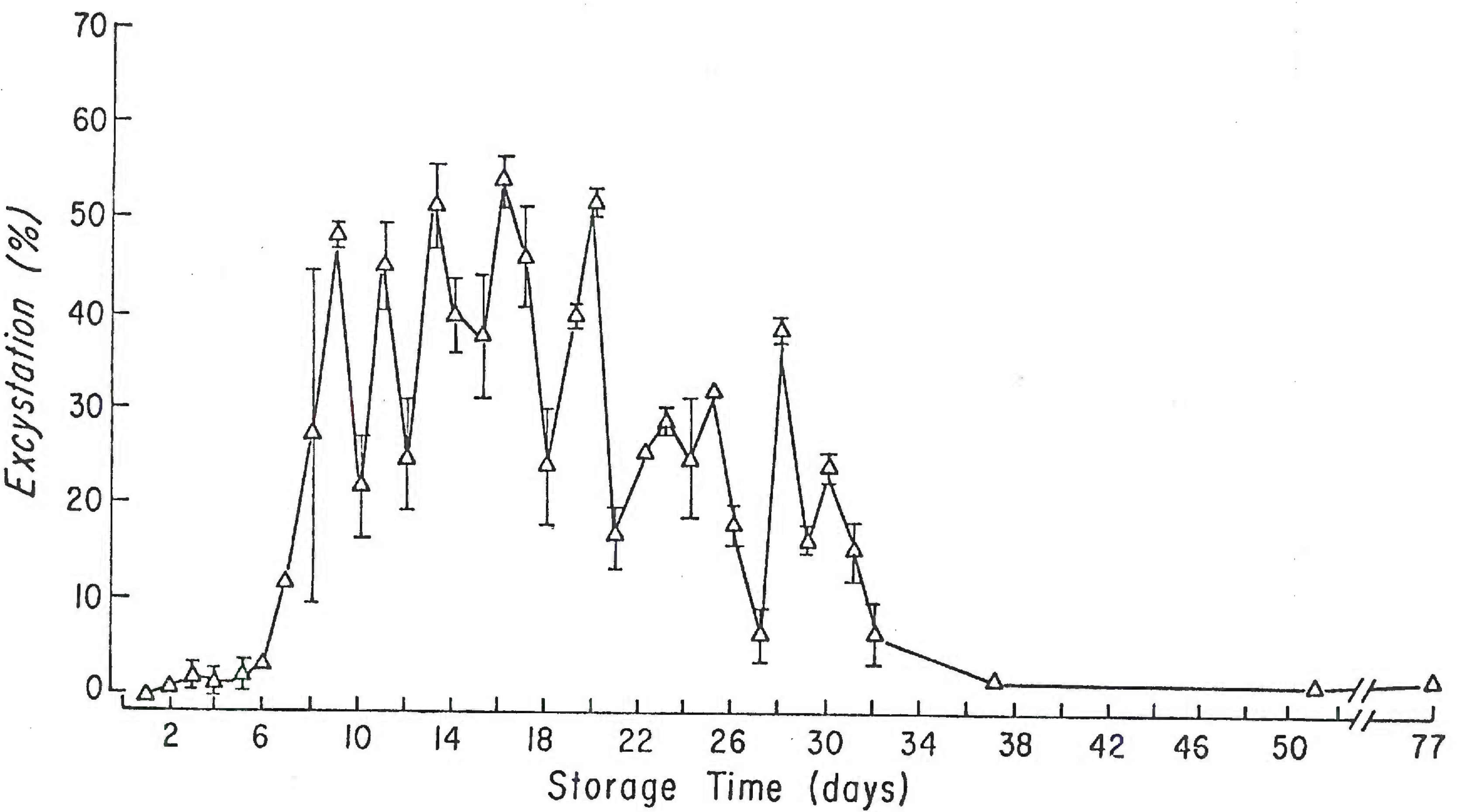

Excystation (%)
70
60
50
40
30
20
10
0
2
6
10
14
18
22
26
30
34
38
42
46
50
77
Storage Time (days)

Figure 8

Effect of storage at 21 C on *Giardia* cyst viability as determined by excystation. Vertical bars represent standard error of the mean.

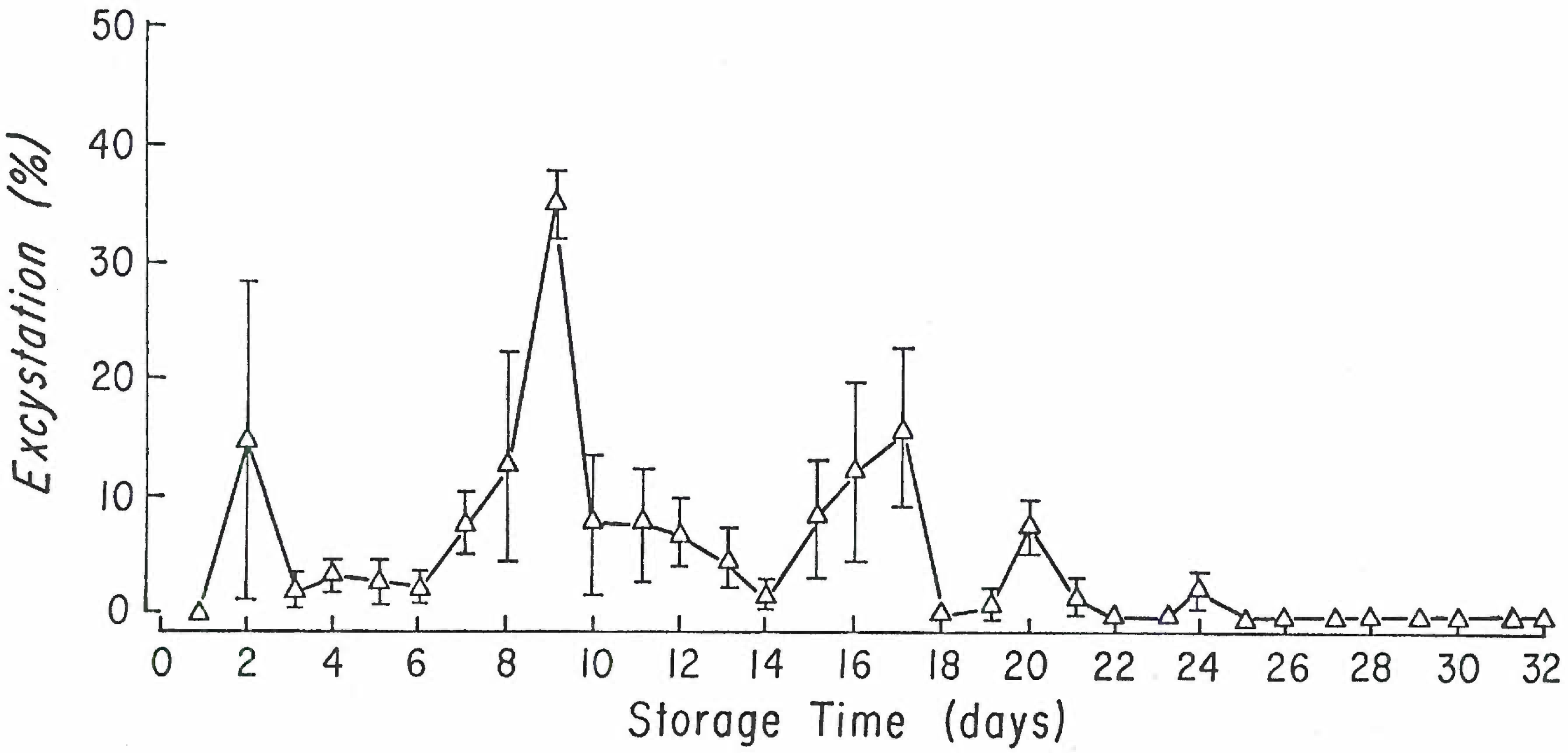

Excystation (%)
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
Storage Time (days)

Figure 9

Effect of storage at 37 C on *Giardia* cyst viability as determined by excystation. Vertical bars represent standard error of the mean.

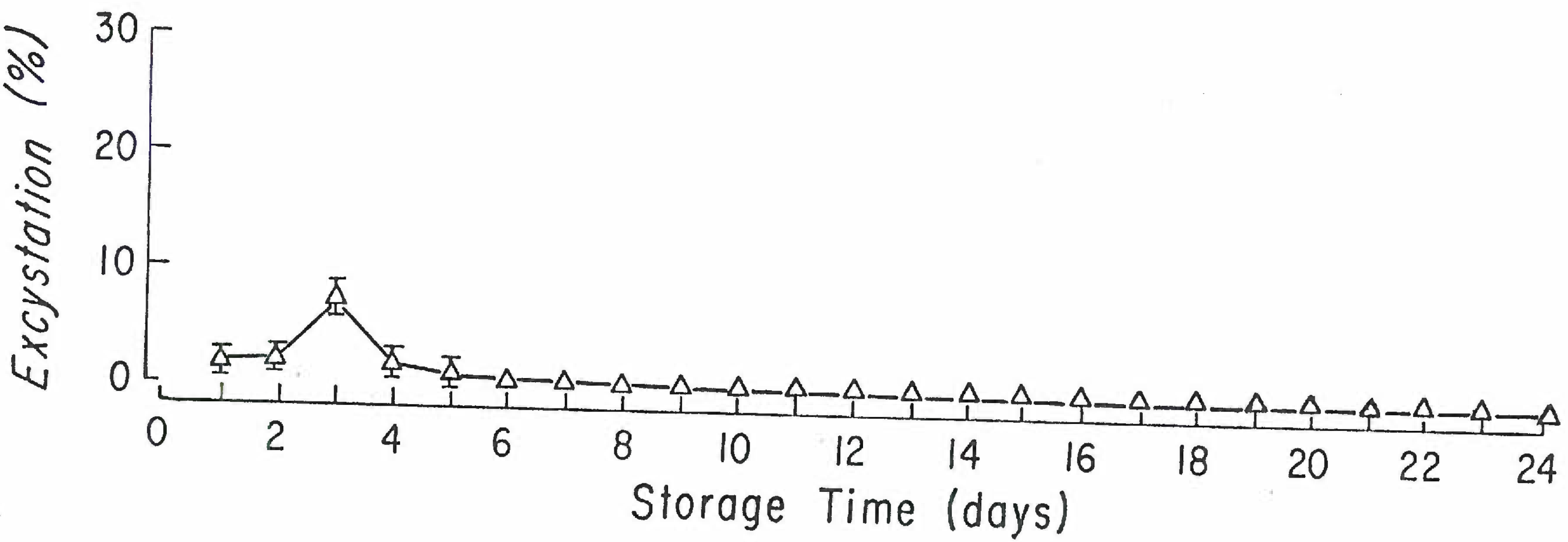

Excystation (%)
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
24
Storage Time (days)

Figure 10

Effect of storage at 37 C on *Giardia* cyst viability as determined by excystation over a 24-hour period.

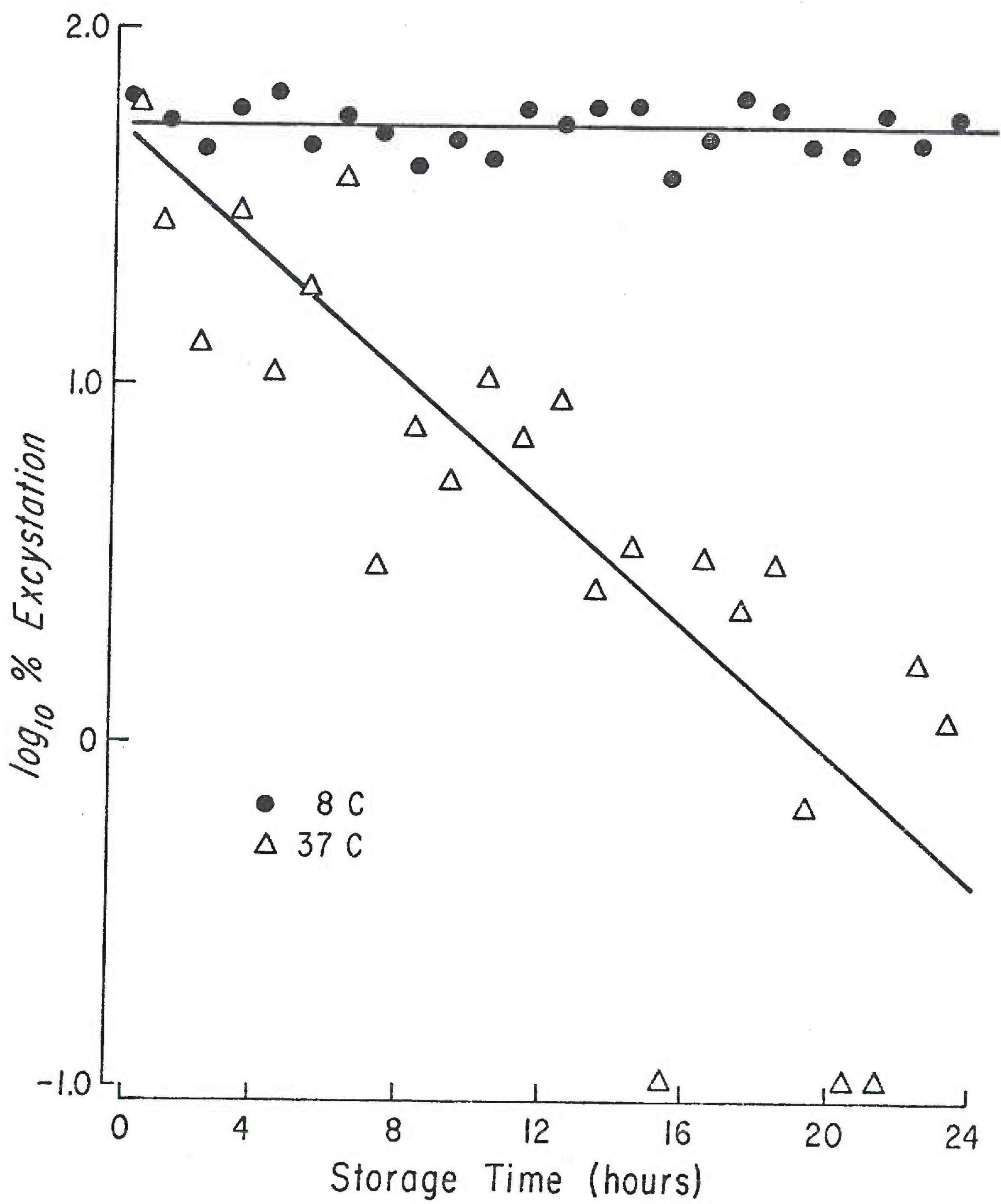
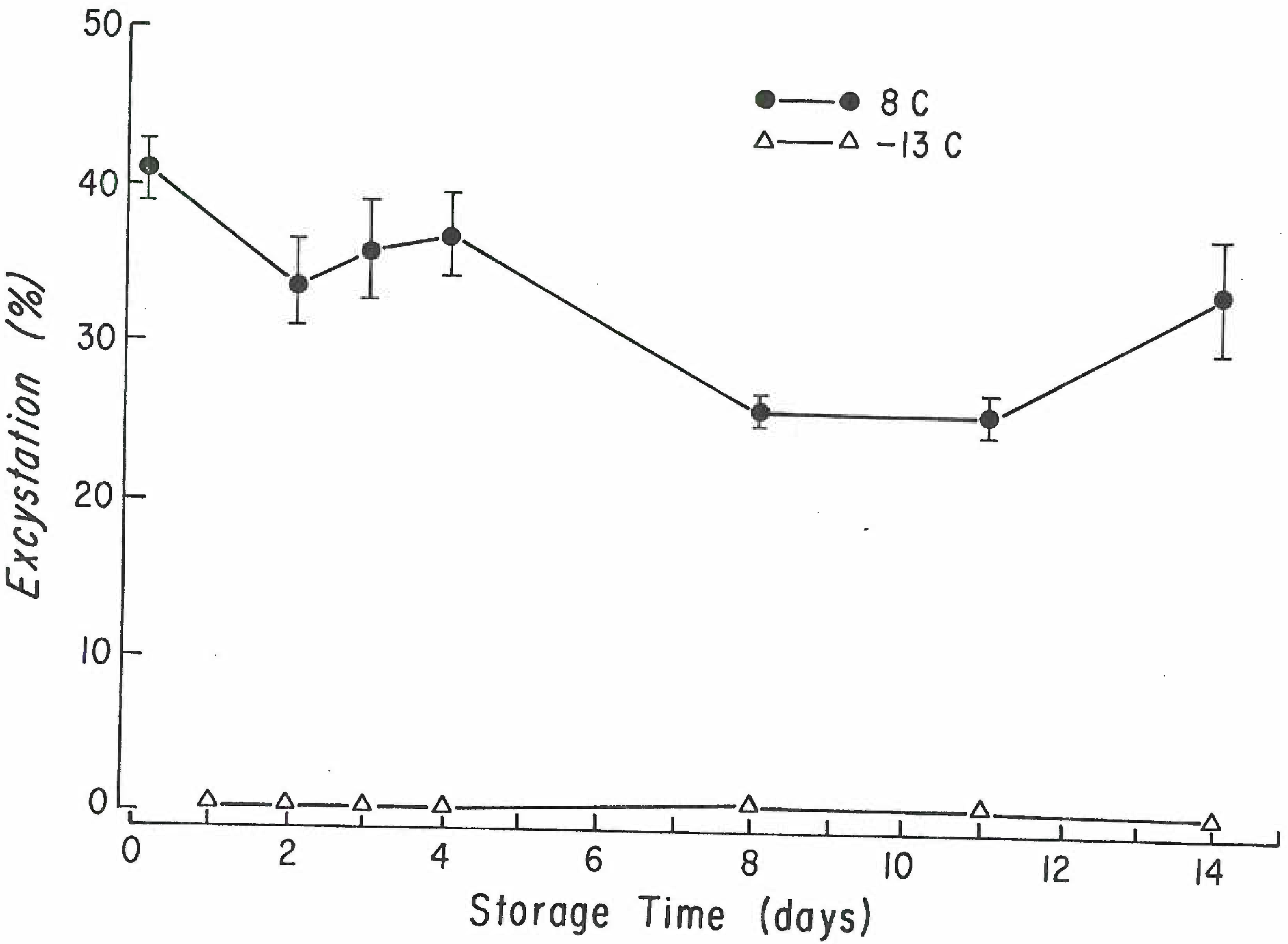

2.0
1.0
0
-1.0
log_{10} % Excystation
8 C
37 C
0
4
8
12
16
20
24
Storage Time (hours)

Figure 11

Effect of storage at -13 C on *Giardia* cyst viability as determined by excystation. Vertical bars represent standard error of the mean.


8 C
-13 C
Excystation (%)
50
40
30
20
10
0
0
2
4
6
8
10
12
14
Storage Time (days)

the $\log_{10}$ percent excystation appeared to decline linearly (r = -0.660) to less than 1 percent after 24 hours. At -13 C storage (Figure 11), the cysts showed almost a total loss of viability within 24 hours, with only 0.2 percent of the cysts surviving to day 11.

An interesting feature of storage at 8 or 21 C was that in all cyst suspensions obtained from fresh fecal specimens, a period of low excystation, varying from two to seven days and followed by an increase in excystation, was observed.

The effects of the HCl and HSP-3 incubation temperatures were examined together. The results of this experiment (Table 6) showed that excystation was dependent upon both the acid and the HSP-3 incubation temperatures. The highest level of excystation occurred when the temperature of both solutions was 37 C. Lower levels of excystation occurred with other temperature combinations, the percentages of excystation increasing as the temperature of the acid, the HSP-3, or both increased. No excystation was induced when the temperature of the HSP-3 was held at 8 C regardless of the temperature of the HCl. No change in the excystation percentages was noted after 18 hours incubation in HSP-3.

D. Post-acid incubation medium. The effect of the composition of the post-acid incubation medium on excystation was examined in two experiments. In the first (Table 7), it was found that significant levels of excystation were induced only in HSP-3 at pH 6.2 and 6.8. Further, excystation was significantly greater in medium at pH 6.8 than at 6.2 ($P < 0.05$).

The second experiment (Table 8) revealed that the levels of excystation in HSP-3 and Hank's Phytone with serum, while not significantly different from each other ($P > 0.05$), were significantly greater than

Table 6

Excystation of *Giardia* at varying temperatures of HCl (pH 2.0) and HSP-3.

Acid temperature (C)	HSP-3 temperature[a] (C)		
	8	21	37
8	0	0.5	1.5
21	0	3.0	5.4
37	0	15.6	100.0

[a] Values represent percentage of excystation with reference to the control tube (HCl 37 C, HSP-3 37 C) which is designated arbitrarily as 100%.

Table 7

Excystation of *Giardia* exposed to HCl (pH 2.0) and transferred into various solutions.

Post-acid solution	pH	% excystation
Water	7.0	0.3
HCl	2.0	0.8
Saline (0.85 M)	7.0	0
PBS	7.0	1.1
HSP-3	0.5	0
	2.0	0
	4.0	0
	6.2	63.3
	6.8	100.0

[a] Percentages of excystation with reference to the control tube (pH 6.8 HSP-3) which is designated arbitrarily as 100%.

Table 8

Excystation of *Giardia* exposed to HCl (pH 2.0) and transferred into component-varied HSP-3 at pH 7.0.

Post-acid solution	Mean[a] % excystation ± S.E.M.
Hanks' Phytone	13.1 ± 1.4
Hanks' Phytone + serum	42.7 ± 3.1
HSP-3	50.9 ± 4.0

[a] Means are derived from an average of 1205 cysts counted in each solution.

excystation in Hanks' Phytone alone ($P < 0.05$).

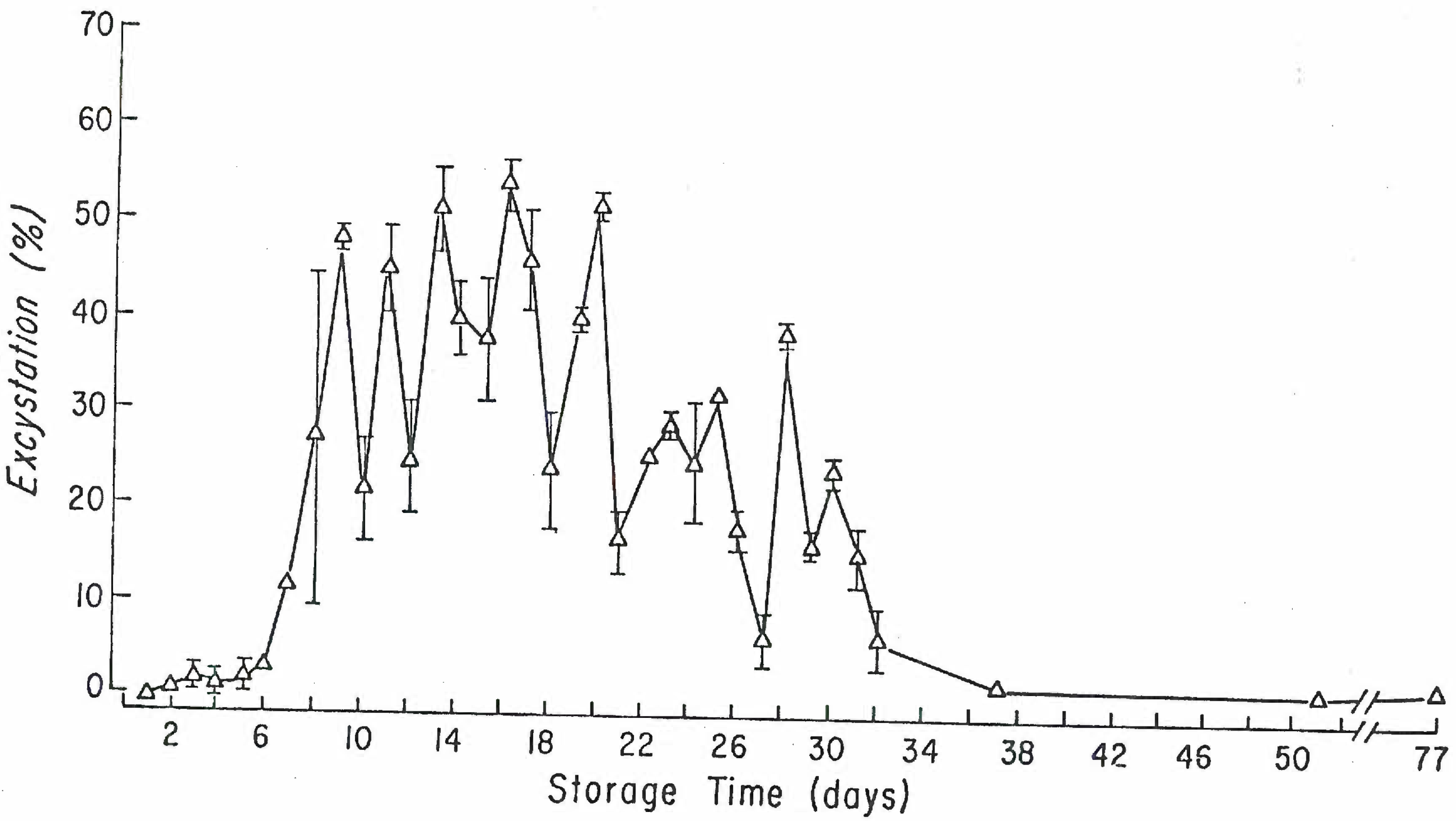
IV. Cyst variation.

A. Maturation, daily variation and viability. Inconsistent results were sometimes found when a single purified cyst preparation was used over a period of days or weeks. This variation appeared to be a result of cyst aging. To study this phenomenon, excystation in several cyst suspensions stored at 8 C was followed over a period of weeks. Figure 12 is the excystation pattern for a single suspension of purified cysts over 11 weeks and is representative of patterns obtained with other samples. This pattern has three important features. As noted previously, an initial four day "lag" period of low excystation was noted, followed by an increase in excystation percentages. Following this lag period, higher levels of excystation were observed until about day 30; during this period large fluctuations in excystation were noted. After the period of elevated excystation, the percent excystation decreased rapidly for approximately a week, and then gradually diminished during the next six weeks, suggesting a loss of viability. Although the specific pattern varied between different cyst preparations, the following characteristics were observed in most suspensions with low-temperature storage: A lag period of one to seven days followed by an increase in excystation over three days preceded a one to four week period of elevated but fluctuating excystation; this was followed by a drop in excystation and an extended period (usually several weeks) of gradually diminishing low-level excystation.

B. Variation in sensitivity to physical environment. The results

Figure 12

Giardia excystation pattern for cysts stored at 8 C for an 11-week period.


Excystation (%)
70
60
50
40
30
20
10
0
2
6
10
14
18
22
26
30
34
38
42
46
50
77
Storage Time (days)

of experiments to determine if cyst sensitivity to the physical environment changes during aging are summarized in Table 9. As the cysts aged, the mean optimum acid exposure time increased from 20 to 70 minutes. From days 34 to 47 the mean optimum time remained at 70 minutes, although it may in fact have been longer since acid exposure times of more than 120 minutes were not tested. The minimal optimum exposure time (see Range, Table 9) did not change markedly during the storage period but remained between 10 and 20 minutes, whereas the maximal optimum exposure time increased from 20 to 120 minutes, resulting in the increasing mean optimum exposure times. Additionally, the mean optimum percentage of excystation showed a similar pattern as observed in previous storage experiments.

V. Excystation as an indicator of cyst viability.

Excystation was compared to dye-exclusion as measures of *Giardia* cyst viability; the results are shown in Figure 13. Eosin-exclusion exhibited an arithmetic decrease with increasing acid exposure time ($r = -0.982$), diminishing from 55.9 percent at zero time to 15.2 percent after 240 minutes. Excystation levels decreased exponentially over the exposure period ($r = -0.988$). Eosin-exclusion consistently indicated higher numbers of viable cysts than could be demonstrated by excystation.

VI. Establishment of axenic cultures.

Excystation was induced in *Giardia* obtained from infected humans, monkeys, dogs, beavers, rats and mice, and cultures were established

Table 9

Optimum time of acid exposure for Giardia excystation with aging of cysts.

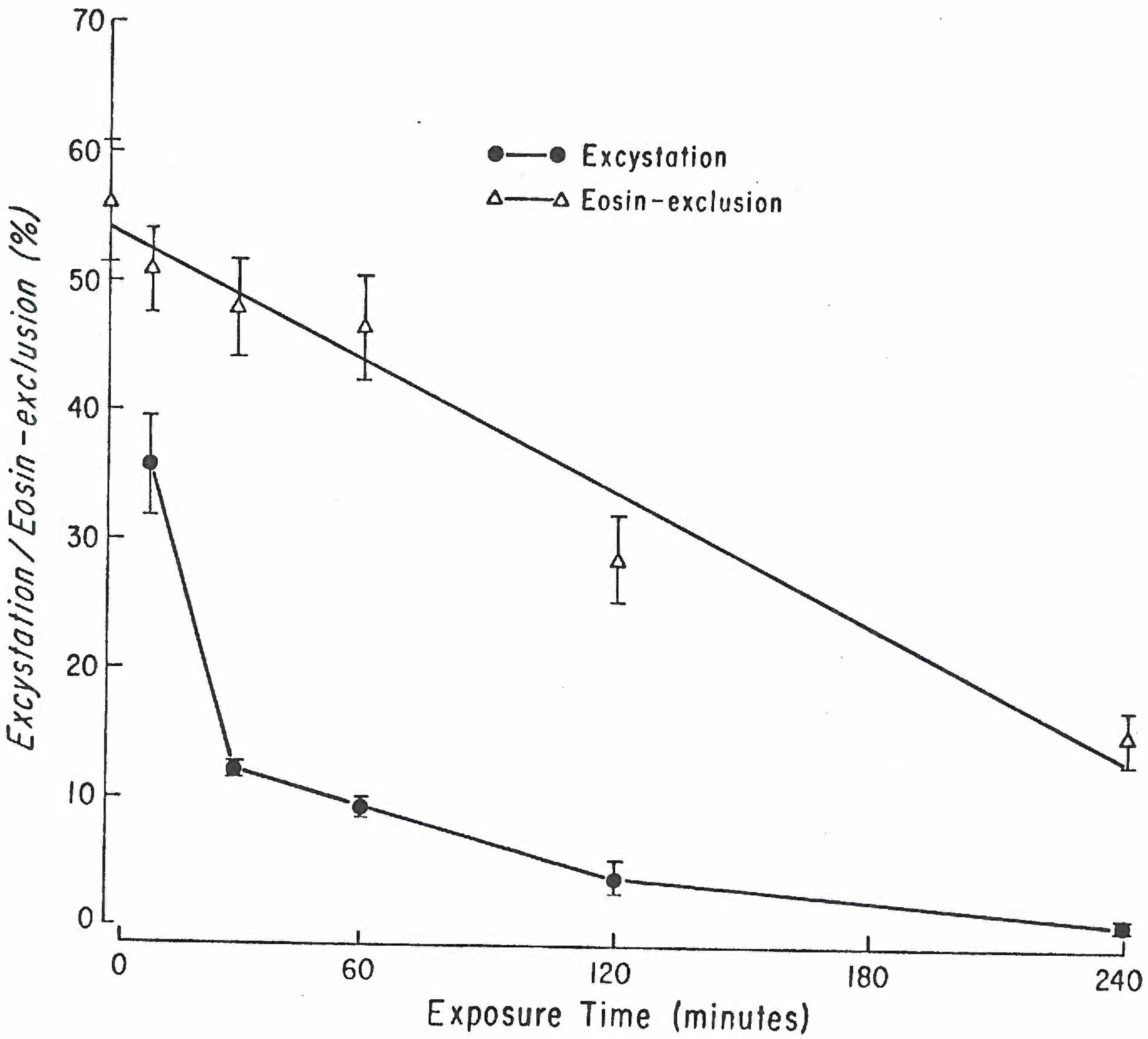
Day	Mean optimum exposure time[a] (min)	Range (min)	Mean optimum % excystation[b] ± S.E.M.
1	20	20	18.1 ± 0.0
5	20	20	20.0 ± 0.0
9	35	10 - 60	8.5 ± 0.6
13	20	10 - 30	11.4 ± 0.4
16	20	10 - 30	8.4 ± 0.7
19	35	10 - 60	7.4 ± 0.4
23	40	20 - 60	6.5 ± 0.6
34	70	20 - 120	3.0 ± 0.4
47	70	20 - 120	1.9 ± 0.2

[a] Average of exposure times at which optimum percentages of excystation were not significantly different from each other on a particular day ($P > 0.05$).

[b] Average of optimum percentages of excystation which were not significantly different from each other on a particular day ($P > 0.05$).

Figure 13

Viability of *Giardia* cysts as measured by excystation and eosin-exclusion with varying exposure time to HCl (pH 2.0). Vertical bars represent standard error of the mean.


Excystation
Eosin-exclusion
Excystation / Eosin-exclusion (%)
70
60
50
40
30
20
10
0
0
60
120
180
240
Exposure Time (minutes)

from excysted trophozoites of cysts from humans and monkeys. These cultures were maintained for up to seven months in HSP-3.

DISCUSSION

The process of Giardia excystation can be divided into four phases. The first is an activation phase during which the trophozoite separates from the cyst wall and begins flexing movements, appearing to rub against one end of the cyst. This phase was first reported in Giardia lamblia by Hegner (30,31,32) and is similar to that seen during the excystation of protozoa such as Entamoeba (42,53), Hartmannella culbertsoni (37), Acanthamoeba castellanii (44) and Eimeria (42), as well as during the excystation of metacercariae of Fasciola hepatica (18) and Paragonimus westermani (69) and the hatching of eggs of Hymenolepis (58, 67), Taenia taeniaeformis (58), and Clonorchis sinensis (24). In some Giardia cysts, excystation did not proceed beyond this initial phase; the trophozoite became quiescent, then expanded, filling the cyst wall. Exhaustion of energy reserves or inability to penetrate the cyst wall could account for the failure to escape. Trophozoite movement in this first phase is probably induced by favorable temperatures since movement was seen in cysts exposed to solutions at 37 C but which were otherwise unfavorable for and incapable of inducing excystation. This is supported by Hegner (30,31,32) who also noted trophozoite movement without excystation in cysts exposed to water or saline at approximately 37 C.

The second excystation phase involves the formation of an aperture in the cyst wall. The mechanism for this phenomenon is unknown but probably involves either a) degradation of the cyst wall by an external environmental factor(s) - "passive excystation" (19), b) disruption of the cyst wall from within by mechanical activity of the trophozoite, or release of a cyst degradative enzyme(s) from the trophozoite or cyst

wall - "active excystation" (19), or c) a combination of passive and active excystation. Observations indicate that the trophozoite alone cannot, either mechanically or enzymatically, disrupt the cyst wall since cysts exposed to certain solutions at 37 C were activated but could not excyst. Therefore, an environmental factor(s) seems to be required. This factor may either a) erode the cyst wall by itself - a chemical reaction such as an acid hydrolysis, b) activate or induce the release of a constitutive enzyme, or c) stimulate the production of an inducible enzyme. Each of the latter two processes would be considered examples of environmentally-induced active excystation - a mechanism which has been proposed for the excystation or hatching of Entamoeba (21), Hartmannella culbertsoni (36), Fasciola hepatica metacercariae (19), Ascaris lumbricoides (23), Hymenolepis diminuta (67), and others (42). Alternatively, it is possible that an environmental factor may act externally in cooperation with an internal enzyme to degrade the cyst - a combination of passive and active excystation.

Except in some forms of aberrant excystation, the aperture was located in one end of the cyst (terminal excystation). The reason for this observation is unknown, but it suggests one of the following: a) the greatest density of receptors for internal or external aperture-producing factors is located terminally; b) a degradative enzyme is localized terminally and is, therefore, ruptured more easily by degradative factors or by the mechanical activity of the trophozoite; or d) a degradative enzyme is released from a region of the trophozoite which is usually in closest proximity to one end of the cyst. Lateral excystation seems to support the latter two concepts; a transposition of an enzyme or enzyme receptors to the side of the cyst is less likely than is the pos-

sibility that the side of some cysts is weaker or thinner than the end, or that the trophozoite may be disoriented in these cysts and release enzyme(s) nearer the side than to the end. The aperture may be formed by the coalescence of several smaller holes produced by a wall-degrading factor. This is supported in part by the observation of several trophozoite cytoplasmic protrusions localized in one area on the surface of some cysts during the third phase of excystation, possibly because the holes did not coalesce. In these cysts excystation was always abortive.

In the third phase the trophozoite begins to emerge from the cyst. The organism appears to force its way through the aperture, suggesting great plasticity of the trophozoite. Excystation may either abort in this phase or proceed to the fourth phase.

In the fourth phase the trophozoite completes its exit through the cyst wall by flexing and flagellar movements. The escape is similar to the process in intestinal protozoan parasites such as *Entamoeba histolytica* (42,53), *Acanthamoeba castellanii* (44) and *Eimeria* (50,56,63), nonparasitic protozoans such as *Blepharisma stoltei* (55), and other parasites such as the metacercariae of the trematode *Paragonimus westermani* (69). As with the first three phases, the fourth sometimes ends abortively. The early stages of trophozoite division were often seen in the fourth phase.

Excystation was usually complete within 5 to 30 minutes after transferring cysts to HSP-3. This is consistent with Hegner's observation of the excystation process *in vivo* (31). The rapidity of the process is suggestive of an enzymatic disruption of the cyst wall, although an external chemical disruption cannot be ruled out. Using the standard procedure with pH 2.0 HCl, excystation levels varied greatly as a reflec-

tion of cyst viability; the highest levels observed ranged from 60 to 65 percent.

Both abortive and aberrant excystation occurred most frequently in cysts of abnormal shape or internal structure, which in turn may be related to a condition of the host such as diet, general health, or immunological response to the infection. Hegner (28) suggested that host diet could influence the course of _Giardia muris_ infections. Tsuchiya (64) found that diet could influence _G. canis_ cyst morphology. Similarly, the general health of the host could have an influence. For example, a febrile reaction or diarrhea due to a concomitant infection by another microorganism might affect _Giardia_ trophozoites and cyst production. It is also possible that abnormal cysts are the result of a host immunological response to the infection. The reason(s) for the occurrence of abnormal cysts and their reduced ability to excyst warrants further investigation.

Observations have shown that as cysts age they progressively lose their ability to excyst. The ratio of abortive to normal excystation also increases with cyst age, due to increasing numbers of abnormal cysts and a concomitant reduction in the numbers of cysts totally excysting. Eventually, no cysts undergo total excystation, and the numbers of cysts undergoing abortive excystation also decreases. In very old cyst suspensions, neither type of excystation is seen.

Many authors (2,17,29,30,31,32,54) have suggested that _Giardia_ excystation might be related to _in vivo_ conditions such as moisture, temperature, gastrointestinal passage time, and exposure to certain digestive fluids. Experiments simulating _in vivo_ conditions suggested that a) an environmental factor(s) is necessary for the induction of excysta-

tion, b) a neutral-pH, isotonic, nutritionally complex solution at physiologic temperature does not induce significant levels of excystation, and c) excystation appears to be pH-dependent.

Experiments investigating the role of pH in excystation suggested that a) excystation is initiated in low-pH solutions, b) the process is dependent on H^+ but is independent of the counter-ion, c) synthetic gastric juice and aqueous HCl, in addition to normal gastric juice, induce excystation at levels which follow similar pH-dependent curves, and d) pepsin and inorganic salts at physiologic concentrations do not influence the levels of excystation significantly.

Several authors have suggested that pH plays a role in *Giardia* excystation. Although Hegner (30,31,32) believed that gastric acidity was detrimental to cysts and unnecessary for excystation, he also thought that pH was probably an important factor in cyst survival and subsequent excystation *in vivo*. Deschiens (17), while studying the effect of gastric acidity on cysts, apparently fortuitously induced the first reported *in vitro* excystation of *Giardia*, using normal gastric juice and HCl. Darby (16) concluded that pH governs the ability of some protozoa to excyst and influences the level and rate of excystation. The pH of the inducing medium also has been reported to play a role in the excystation of *Hartmannella culbertsoni* (37), *Eimeria* (33,63), and *Paragonimus westermani* metacercariae (51,69), and in the hatching of *Taenia* (62) and *Ascaris lumbricoides* (23) eggs. However, *Giardia* excystation differs from that proposed for a number of other parasites (52,65) in being initiated by acid alone and not requiring osmotic change.

Some seemingly contradictory results were observed in these experiments. Although H^+ in concentrations resulting in a pH below 7 appeared

to be necessary for excystation, low levels of excystation sometimes were seen in water and in neutral-pH duodenal-jejunal fluid. In water, only abortive excystation was observed, and always at a level less than 0.5 percent. An aperture must have been formed in these cysts either during the water incubation or during storage. However, cysts were stored in water for weeks with no observed excystation during storage, and it seems unlikely that excystation would occur in water alone since in nature this would mean premature escape outside the host and subsequent death for the trophozoites.

One explanation for the occasional initiation of excystation in neutral solutions is that the cysts were somehow "primed" for excystation while still in storage. For example, yeast and bacterial contaminants or dissolved CO_2 in the cyst suspension could acidify the solution, or microbial contaminants may provide some unidentified excystation-inducing factor(s) which could prime some cysts for excystation. These cysts would remain dormant at the low storage temperature, some eventually becoming non-viable as they aged. Others would survive long enough to be stimulated into excystation when exposed to a favorable temperature, only to be killed by the hypotonic aqueous environment, or not have sufficient energy reserves to totally excyst. This alternative, however, does not explain excystation observed with freshly purified, unstored cysts.

A second, more plausible explanation is that a small percentage of every cyst population is capable of excysting at, or near, neutral pH - a kind of background excystation, consistent with a normal population distribution. Some fecal specimens would have too few of these cysts to be detected by normal sampling procedures. Probably the most active and

viable cyst suspensions would exhibit excystation in water. The fact that only abortive excystation was observed in water can be accounted for by assuming that the trophozoites died during escape when exposed to the water. Yorke and Adams (70) found that excysting Entamoeba histolytica were killed by water, some even before they could escape the cyst wall.

In explaining excystation in duodenal-jejunal fluid, any of the arguments presented for water could be used. However, unlike water, as high as five percent excystation and viable trophozoites were observed. Whether this difference is significant cannot be proven from the available data. Duodenal-jejunal fluid may contain a specific or non-specific factor(s), such as isotonicity, which induces low levels of excystation independent of H^+ concentration. It is also possible that this intestinal fluid, which is the trophozoite's natural habitat, enhances the excystation of cysts which have been primed during storage. Alternatively, and most likely, background excystation may be enhanced in duodenal-jejunal fluid; instead of being killed as they are in water, the trophozoites are in a highly favorable environment which allows excystation of all trophozoites capable of doing so under neutral-pH conditions.

These observations may explain why persons with hypochlorhydria and achlorhydria apparently have contracted giardiasis (25). They also explain why some investigators such as Armaghan (2) found excystation in cysts introduced directly into the intestinal tract of animals, bypassing the stomach. In any case, these findings do not vitiate the role of pH in inducing and influencing the process of excystation.

In the previous set of experiments the standardized excystation

procedure was utilized on cysts stored at 8 C. Inherent in this procedure were several variables which required investigation in detail to determine their effects on excystation. These variables included pH, time of acid exposure, cyst storage temperature, temperature of the acid and HPS-3 incubation solutions, and composition of the post-acid incubation medium.

Experiments investigating the influence of pH and time of acid exposure on excystation suggested a pH-time dependency, similar to that reported with other protozoa (16,31,37,63). The linearity of the plotted data indicates that the induction of excystation is a first-order reaction, consistent with either a H^+ concentration-dependent acid hydrolysis or an enzymatic degradation of the cyst wall. The rate of an acid hydrolysis would depend upon the H^+ concentration - the lower the pH, the shorter the time. Likewise, the activation or release of a constitutive or an inducible enzyme in the presence of H^+ would both depend for activity upon the intra-cystic pH, which would in turn depend upon the rate of entry of H^+ into the cyst. The time dependency of the entry of H^+ suggests either a) a passive diffusion, the rate of which is dependent upon the external H^+ concentration of b) an active transport with competition for H^+ by more than one trophozoite enzyme. The similarity between the results at pH 0.5 and 2.0 suggests that these H^+ concentrations are optimal for excystation.

The observation of minimal, maximal and optimal excystation times at specific pH values can be explained by considering the results at pH 2.0. At five minutes, only the most "sensitive" cysts have been stimulated to excyst, resulting in 28 percent excystation; most are still in the process of cyst wall disruption. Between 10 and 30 minutes, the

majority are prepared to escape the cyst wall when transferred into HSP-3; this is the optimum acid exposure time for the cysts. Longer exposure times become increasingly unfavorable to the trophozoites. These observations suggest that once a cyst is exposed to H^+ for a certain minimum time it becomes committed to excystation, and will attempt to excyst in whatever environment it is; if the environment is adverse, the trophozoite will die. There are three possible explanations for the adverse effect of extended acid exposure. Once the cyst is committed to excystation and the wall disrupted, some trophozoites may be killed within the cyst wall by the acid environment, since pH values below 6.2 are detrimental to the survival of trophozoites (46). Trophozoites may require an environmental factor(s), possibly nutritional in nature, in order to escape from the cyst once the wall is disrupted; these factors could be absent in aqueous HCl, but present in HSP-3. During a prolonged acid exposure, trophozoites could be activated metabolically, their energy reserves so as not to be capable of escaping from the cyst when transferred into HSP-3. This is supported by one form of abortive excystation in which trophozoite movement was induced when cysts were exposed to certain solutions at 37 C, but the activity subsequently ceased and the trophozoite expanded to fill the cyst.

The pH-time experiment suggested that at pH 0.5 and 2.0, all of the cysts capable of excysting have done so, resulting in high mean optimum percentages of excystation. At pH 4.0 and 6.2 the conditions are not conducive to the induction of excystation in as large a percentage of cysts, resulting in low levels of excystation. The reason for this excystation preference is not easily explained. Cysts exposed to pH 4.0 and 6.2 for long enough periods *in vitro* might well be expected to have

optimum excystation levels comparable to those at pH 0.5 and 2.0. All four HCl solutions are similar in not containing any nutrients which might be necessary for excystation. Moreover, acid killing of the trophozoites within the cysts should actually be less at higher pH values since these are close to the optimum growth pH (6.4 to 7.2; [27,46]) than are the more concentrated solutions. One explanation is that the degradation of the cyst wall at high pH takes an extended period of time during which the trophozoite energy reserves are depleted, as suggested previously. This is possible with either the acid hydrolysis or enzyme degradation theory. In the case of an enzyme-mediated escape, the enzyme may not be as active at high pH values as it is at the more acidic conditions, and therefore only cysts most sensitive to the hydrogen ion will excyst at pH 4.0 and 6.2. Because of reduced enzyme activity at high pH, the time required for cyst wall degradation might be extended, resulting in depletion of trophozoite energy reserves. These alternatives do not exclude the possiblity that an environmental factor(s) (other than H^+) may be needed for excystation.

The pH-time experiment also revealed that the time interval during which optimum excystation occurred was shortest for cysts in the most concentrated acidic solution and longest in less concentrated solutions. At low pH cyst wall disruption would take a short time for most cysts, and many of these trophozoites would not complete excystation if acid exposure was extended. This factor would result in a narrow optimum time interval. In a relatively mild acid solution cyst disruption would take longer, the trophozoites would survive longer exposure periods, and a longer optimum time interval would result.

In storage experiments, cysts survived best at 8 C. One possible

explanation for reduced cyst survival in water at 21 and 37 C is that the trophozoites may have been activated when exposed to these temperatures, but due to an insufficient H^+ concentration, could not excyst and ultimately depleted their energy reserves. This hypothesis is suggested by the exponential decrease in cyst viability over 24 hours incubation at 37 C. Since a temperature of 37 C is not itself lethal to cysts, it must cause some other effect, such as stimulating trophozoite activity, which is ultimately detrimental to cyst viability. These observations suggest that an extended period of intestinal passage in the infected host may decrease cyst viability; cysts rapidly eliminated from the host may have the greatest excystation potential. At 8 C, cyst metabolic rate is at a low level and energy depletion is minimal. At first, this argument seems inconsistent with the low cyst survival observed at -13 C. However, the fact that there was some cyst survival at -13 C suggests that the freezing process and not the low temperature may have been responsible for cyst death.

The above observations indicate that cysts are more likely to survive in cool water, such as mountain streams, than in warm water. Relatively few survive freezing. These results are similar to those of Chang and Fair (11) who reported that the period of *E. histolytica* survival in water at 8, 21 and 37 C was approximately 36, 9 and 2 days respectively. These researchers also used excystation as the criterion for viability. More recently, Grant and Woo (26) found that *Giardia simoni* cysts retained infectivity better at 5 C than at 24 C, which in turn was better than 36 C. The harmful effect of freezing on other protozoan cysts has been reported. Neal (48,49) found that *Entamoeba* cysts maintained viability from 16 to 46 weeks at 4 C, but were rendered

non-viable in less than a week when frozen in liquid nitrogen.

The temperature of both the acid solution and the subsequent excystation medium also influenced excystation levels. It was found that when the temperature of both solutions was 37 C, the level of excystation was more than six times greater than at the next most favorable temperature combination (37 C acid, 21 C HSP-3). In addition, the percentage of excystation increased as the temperature of the acid, HSP-3, or both increased to 37 C. These results indicated that the excystation of Giardia proceeds best at physiological temperatures. When the temperature of the HSP-3 was held at 8 C, no excystation occurred regardless of the acid temperature, suggesting that the HSP-3 temperature was more critical than the acid temperature. Temperature has been shown to be an important factor in the excystation or hatching of many parasites, most of which show a preference for 37 C.

The effect of the composition of the post-acid incubation medium on excystation was examined in two experiments. Significantly higher levels of excystation were observed in HSP-3 at pH 6.8 than in pH 6.2 HSP-3. Both induced significantly greater levels of excystation than other solutions, suggesting that the process was influenced by both the pH and the composition (exclusive of H^+) of the post-acid medium. Completion of the excystation process was not seen in any solutions below pH 6.2, presumably due either to the adverse effect of the acidity (as in acidified HSP-3), or to the lack of some necessary factor(s). Lack of excystation in acidified HSP-3 lends support to previous indications that low pH may adversely affect intra-cystic trophozoites. The findings indicate that while H^+ activates excystation, subsequent transfer to a favorable medium is required for completion of the process. These results do not sub-

stantiate the observations of Deschiens (17) who observed apparently excysted (albeit dead) "vegetative forms" after placing Giardia intestinalis cysts in aqueous HCl and gastric juice, or of Armaghan (2) who reported that G. muris trophozoites survived gastric passage in the rat.

The results of the component-varied HSP-3 experiment suggested that serum in the post-acid incubation medium stimulated, but was not required for, excystation. The nature of this stimulatory effect is not known, but it may be osmotic, or it may be due to some specific factor(s) in the serum. Danciger (14) found that serum contains unspecified factors which are necessary for the in vitro cultivation of Giardia trophozoites. These same factors may aid excystation. The NCTC-135, present in HSP-3 but not in Hanks' Phytone + serum, did not enhance excystation to any significant degree, suggesting that NCTC-135 either a) contains no factors contributing to excystation, or b) contains them in concentrations too low to produce an observable effect. The only complex solution examined as a post-acid incubation medium for Giardia was HSP-3, but other solutions may also prove satisfactory for inducing excystation. The metacercariae of Paragonimus westermani have been shown to excyst when exposed to a medium consisting of an isotonic salt solution at 40 C and pH 9, following initial exposure to an acid medium of pH 3 (51,69).

In some experiments, fluctuations in the percentage of excystation were observed over a period of time using a single suspension of purified cysts. This phenomenon was examined in greater detail. Freshly excreted cysts stored at 8 C exhibited an initial period of low excystation which varied from one to seven days, followed by an increase in excystation. The consistency with which this "lag" period was observed in this and other experiments at 8 and 21 C, suggests that Giardia cysts

require a maturation period before reaching their full excystation potential. The reason for this maturation period is unknown but may be related to nuclear division or other physiological changes in preparation for excystation and cell division. Although Boeck (4,5) and Hegner (30) suggested that Giardia cysts require a maturation period, this is the first quantitative evidence of this phenomenon. Cyst maturation has been reported for other protozoa such Acanthamoeba castellanii (10,22) and Entamoeba histolytica (70).

Following the initial period of low excystation, the levels of excystation increased steadily over about three days, followed by a period of several weeks during which maximal excystation percentages were seen. It is of interest that this period was also characterized by large daily fluctuations in excystation percentages. The source of this variation is due in part to variability in sampling. However, it may also be a result of variation among cysts. A single fecal specimen could contain cysts with a wide range of responsiveness to the excystation procedure, and of ages (with corresponding maturities) from a few hours to more than two days.

Levels of excystation decreased over a period of two days to low levels, then gradually diminished further during the ensuing several weeks. This decrease in excystation was probably due to a depletion of cyst energy reserves, resulting in an inability to excyst and subsequent death. The two day period of rapidly decreasing excystation levels may coincide with the depletion of energy reserves. This is consistent with the suggestion that cysts in a fecal specimen may vary approximately two days in age and maturity. Although some cysts remained viable for weeks, excystation was low and in almost all cases abortive, suggesting that the

inability to escape completely from the cyst was due to the depletion of energy reserves.

Another experiment was designed to determine if cyst sensitivity to physical variables changed as cysts aged. One variable, the optimum time of incubation in pH 2.0 HCl, was examined. The results suggest that cysts do indeed vary in sensitivity to the physical environment as they age. The mean optimum exposure time increased by 50 minutes over the storage period, while at the same time the mean optimum percent excystation decreased by 90 percent. These data suggest that as the ability to excyst decreases with advancing cyst age, the sensitivity of the remaining viable cysts to the physical environment also decreases.

One experiment was performed to investigate a practical application of *in vitro* excystation: Its use as a determinant of cyst viability. Excystation was compared to dye-exclusion - a widely accepted method for determining cyst viability. Although both eosin-exclusion and excystation indicated an overall decrease in cyst viability with increasing acid exposure time, the results suggested a difference in what each of these methods was actually measuring. Excystation decreased logarithmically, indicating that the rate of killing of the trophozoites within the cysts was first-order. Killing was probably effected by prolonged acid exposure as suggested by previous experiments. On the other hand, eosin-exclusion decreased arithmetically and indicated cyst viability percentages which were consistently higher than indicated by excystation. This finding suggests that the eosin-excluding barrier, possibly the trophozoite cell membrane, remains intact for a time after the cysts are rendered non-viable. Grant and Woo (26) also observed that apparently non-viable *Giardia* cysts could exclude eosin. Eosin-exclusion then would

measure the ability of the cyst to exclude dye, whereas excystation measures the ability of trophozoites to escape the cyst wall. If one considers the ability of a trophozoite to infect a new host as the true measure of viability, then excystation is a more reliable measure of viability since escape of the trophozoite from the cyst is prerequisite to infection. Although eosin-exclusion has been used extensively for determining the viability of protozoan cysts including *Giardia* (4,5,9), some authors (11,26,72) have criticized its reliability, preferring rather *in vitro* excystation or infectivity as their criteria for protozoan cyst viability. The results of this experiment support the criticisms of these latter authors.

The excystation profile for this experiment was consistent with that seen for previous experiments, notably the pH vs. time study (see Table 5). The results showed that the cysts must be exposed to acid for a minimum length of time (less than 10 minutes in this experiment) in order for excystation to be initiated. Once cysts are exposed to acid for this minimal time, they become committed to excystation, and death will ensue if they are not transferred into a favorable medium within a few minutes. If there is a minimal exposure time for initiation of excystation and trophozoite death occurs with prolonged exposure, then there must be an optimum exposure time at which the highest excystation levels will occur.

SUMMARY AND CONCLUSIONS

Giardia excystation was induced *in vitro* by exposing cysts to gastric juice followed by transfer into growth medium HSP-3. The escape of trophozoites from the cyst wall was observed, photographed, and axenic cultures were initiated from these trophozoites.

Investigation of the factors responsible for the initiation of excystation revealed that the H^+ concentration was responsible for the initiation of excystation. The levels of excystation achieved were dependent upon pH. Salts and pepsin did not influence the process.

The time of acid exposure needed to achieve highest excystation percentages was found to vary according to pH. Optimum levels of excystation occurred at pH values below 3.5 and exposure times less than 60 minutes.

Excystation was a temperature-dependent process; cyst storage temperature as well as the acid and HSP-3 temperatures influenced excystation. Optimum survival occurred when cysts were stored at temperatures between 0 and 8 C; freezing was detrimental to cysts. Excystation proceeded best when the acid and HSP-3 were both at 37 C.

The composition of the post-acid incubation medium influenced excystation. Excystation was enhanced by the addition of serum to the post-acid medium, but inhibited if the medium was acidified. Optimum levels of excystation were achieved when cysts were transferred from acid into either HSP-3 or Hanks' Phytone with serum, both at pH 6.8 to 7.0.

Observations of excystation with long-term cyst storage at 8 C indicated that cysts undergo an excystation pattern characterized by a) a

maturation period of one to seven days during which low-level excystation occurred, followed by b) an increase in mean excystation levels lasting from 1 to 4 weeks, then c) a rapid drop in the percentages of excystation followed by a period of gradually diminishing excystation lasting several weeks. Additionally, cysts became less sensitive to H^+ with aging; longer exposure times were required to achieve highest excystation percentages.

Comparison of excystation and eosin-exclusion as measures of cyst viability revealed that dye-exclusion consistently indicated greater numbers of viable cysts than indicated by excystation, suggesting that excystation is a more accurate indicator of cyst viability than is dye-exclusion.

The factors found in these experiments to be most conducive to excystation suggest that *Giardia* excystation proceeds best under those conditions which exist in the host stomach and small intestine.

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