Effect of Sodium Hypochlorite Concentrations on Selected Genera of Nematodes

ROBERT P. ESSER
Division of Plant Industry, Gainesville, Florida

ABSTRACT: Eleven genera of nematodes were immersed in undilute and three dilutions (1:5, 1:10, and 1:15) of NaOCl until they were no longer identifiable. At a 1:10 dilution or less most nematodes were unidentifiable in less than 7 min. Larvae withstood the effects of NaOCl the longest while males were most susceptible. Nematode exposure in a 1:5 dilution of NaOCl for 10 min is considered an effective rate for disinfection purposes.

Sodium hypochlorite (NaOCl) dilutions have been used to surface sterilize nematodes (*Radopholus similis*) (Feder and Feldmesser, 1955) and nematode eggs (*Rhabditis*) (Briggs, 1946). It has also been used to dissolve the gelatinous matrix enclosing *Meloidogyne* sp. eggs (Tyler, 1938). The albuminoid and chitinous layers of *Ascaris* egg shells have been dissolved with NaOCl to obtain aseptic eggs (Guevara Pozo, 1953). *Heterodera glycines* larvae have been treated with NaOCl to study cuticular layering (Hirschmann, 1959).

No reference was found concerning the effect of NaOCl on animal tissue culminating in disintegration of the tissue. Apparently NaOCl acting as a strong oxidizing agent breaks the sulphide bonds of the tissue, altering the orientation of the tissue and subsequently its tertiary structure. Hydrolysis also occurs, possibly accelerating the oxidation process. Almost any animal tissue subject to an alteration in its tertiary structure is also subject to dissolution.

In the operation of a regulatory diagnostic nematology laboratory where numerous soil samples are processed, a problem of utmost importance is the maintenance of strict sanitary procedures to prevent nematode contamination of processing substrates and equipment.

Should a nematode contaminant with regulatory status pass from substrate or equipment to a soil sample from whence it did not originate, serious economic ramifications could erroneously result.

Nematodes that desiccate on glass collapse and adhere to the glass. Reconstitution in water and examination under the microscope showed that many such nematodes could still be identified to genus.

Persistence of dead nematodes on laboratory glass was tested by allowing mixed populations of nematodes to air dry for several weeks on glass slides, then washing the slides under a hard spray of water. Some nematodes washed off, but many remaining ones were easily identifiable to genus. Further drying and washing of the same slides removed more but not all nematodes.

The primary objective of this study was to determine a concentration of NaOCl that will disintegrate a nematode until it becomes taxonomically unrecognizable in a minimal time.

A secondary purpose was to evaluate the gross effects of NaOCl on various types of nematodes.

The ultimate goal was to establish a known dilution of NaOCl that could serve as an effective decontaminant for laboratory equipment and substrates.

Materials and Methods

Sodium hypochlorite (active ingredients 5.25%) available commercially as household bleach was used undiluted and diluted with tap water at ratios of 1:5, 1:10, and 1:15.

Dropper bottles were filled to capacity with test solutions to reduce deterioration of the NaOCl and fresh solutions were made every 2 weeks.

Nematodes to be tested were selected only if in excellent physical condition and in an active state fresh from soil or root extraction. Bacteriophagous, myceliophagous, and phytoparasitic nematodes from either or both of the classes Adenophorea or Secernentea were used.

A drop of the test solution was placed on a microscope slide and the slide placed under a 150 magnification of the compound microscope.

Table 1. Means of four specimens per genus (or sex) exposed to dilutions of NaOCl expressed in time elapsed (to the nearest minute) following immersion of the specimens in the test solution. Rupture: time after immersion until cuticle ruptures. Unidentifiable: indicated time after immersion until the specimen can no longer be reliably identified to genus.

Nematode genus	Sex	Rupture (Dilution rate of NaOCl)				Unidentifiable (Dilution rate of NaOCl)			
		Undilute* Min	1:5 Min	1:10 Min	1:15 Min	Undilute Min	1:5 Min	1:10 Min	1:15 Min
Aphelenchus sp.	♀	1	1	0	0	2	2	0	0
Helicotylenchus sp.	♀	<1	1	1	5	1	2	3	19
Helicotylenchus sp. (molting)	♀	<1	1	0	0	2	3	0	0
Hemicycliophora sp.	♀	2	5†	0	0	9	35	0	0
Heterodera glycines	Cyst	3	7	20	12	9	21	39	42
Hoplolaimus sp.	♀	<1	2	4	9	2	4	7	14
Meloidogyne sp.	♂	<1	1	2	9	1	1	3	13
Meloidogyne sp.	Larvae	1	3	3	12	2	4	4	13
Pratylenchus sp.	♀	<1	1	3	10	1	2	6	16
Radopholus similis	♀	<1	1	5	70	1	2	6	74
Radopholus similis	♂	1	1	2	0	1	1	3	0
Radopholus similis (molting)	♀	12	12	0	0	13	15	0	0
Rhabditis sp.	♀	1	4	3	10	1	4	4	11
Tylenchulus semipenetrans	Larvae	<1	11	32	27	1	12	32	25
Xiphinema americanum	♀	<1	<1	1	1	1	1	3	4

* Based on 5.25% solution of NaOCl.
† Three specimens.

The time in minutes and seconds was recorded when the nematode was immersed in the test drop. The specimen was observed continuously until the body ruptured, then the time elapsed recorded. When the specimen could no longer be identified to genus the final time was recorded.

Each test was replicated 4 times in each test solution.

Results

Effect of NaOCl on specific genera: These data are presented in tabular form in Table 1 and the important considerations of each genus will be pointed out.

Rhabditis sp. (females): Habit: Bacteriophagous

Dilution rate	Range Rupture Min	Sec	Min	Sec	Unidentifiable Min	Sec	Min	Sec
Undilute		25	1	25		50	1	55
1:5	2	30	4	05	3	00	4	25
1:10	1	05	7	00	1	25	7	10
1:15	1	20	20	15	1	30	19	50

Rhabditis sp. differed in morphology from all other nematodes tested in having a large open oral aperture and by shrinking severely upon immersion. Disintegration was slow, gradual, and overall.

Aphelenchus sp. (females): Habit: Mycelio-phagous

Dilution rate	Range Rupture Min	Sec	Min	Sec	Unidentifiable Min	Sec	Min	Sec
Undilute		30	1	50	1	45	2	10
1:5		40	1	35	1	10	2	25

Aphelenchus sp. disintegrated slowly with no dramatic effects. Specimens in the test population were not sufficient in proper condition to complete the series.

Hemicycliophora sp. (females): Habit: Ecto-Phytoparasitic

Dilution rate	Range Rupture Min	Sec	Min	Sec	Unidentifiable Min	Sec	Min	Sec
Undilute	1	25	4	00	5	05	12	50
1:5	5	00	5	45	34	40	36	10

This nematode differed from all others tested in possession of a sheath (two cuticles) which one would assume to provide special protection in its environment. Such has been indicated in predation studies (Esser, 1963). In the undilute solution the body inside the sheath ruptured in all four specimens indicating the sheath was penetrated easily by the solution.

In two of four specimens the bodies were ejected forcibly from the sheath. In the 1:5 solution the inner body cuticle dissolved slowly prior to the outer sheath dissolving. Air bubbles emerged orally and from the vulva. The body separated from the sheath attachments first at the head and then the vulva. This was the most resistant nematode tested in the undilute and 1:5 solution.

Xiphinema sp. (females): Habit: Ecto-Phytoparasitic

Dilution rate	Range: Rupture Min	Sec	Rupture Min	Sec	Unidentifiable Min	Sec	Unidentifiable Min	Sec
Undilute		10		20		35		55
1:5		25		30		55	1	40
1:10		35		55	2	00	3	15
1:15		50	1	05	3	45	4	00

Xiphinema sp. differs from all other nematodes tested in having hypodermal glands throughout the body length allowing easy access of chemicals into the body. Death and disintegration was very rapid. Undilute solutions disintegrated the specimens very fast, the entire cuticle peeling off. The basal odondostylet dissolved in 1½ minutes.

In the 1:5 dilution the body ruptured at many points, the body contents gushed out, and the cuticular layers peeled off. At 1:10 the body ruptured at fewer points and the contents oozed out slowly.

Hoplolaimus sp. (females): Habit: Ecto-Phytoparasitic

Dilution rate	Range: Rupture Min	Sec	Rupture Min	Sec	Unidentifiable Min	Sec	Unidentifiable Min	Sec
Undilute		30		45	1	10	2	00
1:5	1	35	2	05	2	55	4	40
1:10	3	55	5	30	6	10	9	05
1:15	6	55	10	30	13	25	16	50

This is a relatively large nematode with a thick cuticle. Undilute solution caused the cuticle to peel off its entire length and disintegrate. At a rate of 1:5 the cuticle ballooned out and separated into two layers. Many vesicles appeared between the layers. The cephalic framework intact with stylet detached itself (also in undilute test). The body burst in a number of places and peeled off. In 1:10 solutions the cuticle peeled off slowly in pieces. Some tails split open and the contents flowed out.

Helicotylenchus sp. (females): Habit: Ecto-Semi-Endo-Phytoparasitic

Dilution rate	Range: Rupture Min	Sec	Rupture Min	Sec	Unidentifiable Min	Sec	Unidentifiable Min	Sec
Undilute		15		30		50	1	45
1:5		55	1	10	1	30	2	15
1:10		30	2	00	2	00	3	20
1:15			8	00	2	10	54	10

Helicotylenchus sp. ruptured and disintegrated undramatically. Ruptures occurred at various points on the body. In one molting specimen the cuticle dissolved in less than 5 sec; in the other it merely detached. One specimen persisted in an identifiable state for 54 min at the 1:15 rate without rupturing. This widened the above range considerably, also affecting the series mean.

Heterodera glycines (mature cysts): Habit: Semi-Endo-Phytoparasitic

Dilution rate	Range: Rupture Min	Sec	Rupture Min	Sec	Unidentifiable Min	Sec	Unidentifiable Min	Sec
Undilute	1	45	3	45	5	05	12	45
1:5		40	20	00	8	35	32	25
1:10	7	00	31	00	34	05	44	05
1:15	7	05	21	00	28	15	62	10

This nematode differs from all others in that the swollen female hardens into a protective cyst containing eggs. Undilute rates of NaOCl caused many air bubbles to emerge from the inside of the cysts (Fig. 1-A). In a short time the outer cyst opened and the eggs fell out (Fig. 1-B). Egg contents quickly deteriorated (Fig. 1-C). Some eggs burst in 18 min; in all others observed only the outer shell dissolved. The vitelline membrane, being insoluble in NaOCl (Chitwood, 1938), persisted (Fig. 1-D). Evolving air bubbles decreased with dilution with very few being produced in the 1:10 and 1:15 tests.

Tylenchulus semipenetrans (larvae): Habit: Semi-Endo-Phytoparasitic

Dilution rate	Range: Rupture Min	Sec	Rupture Min	Sec	Unidentifiable Min	Sec	Unidentifiable Min	Sec
Undilute		20		50		30	1	20
1:5	1	35	34	40	1	55	34	45
1:10	24	40	50	10	24	50	50	10
1:15	12	50	48	40	14	05	40	40

Disintegration was gradual and uneventful. A relatively fast easy kill in undilute was followed by very slow reduction to unidenti-

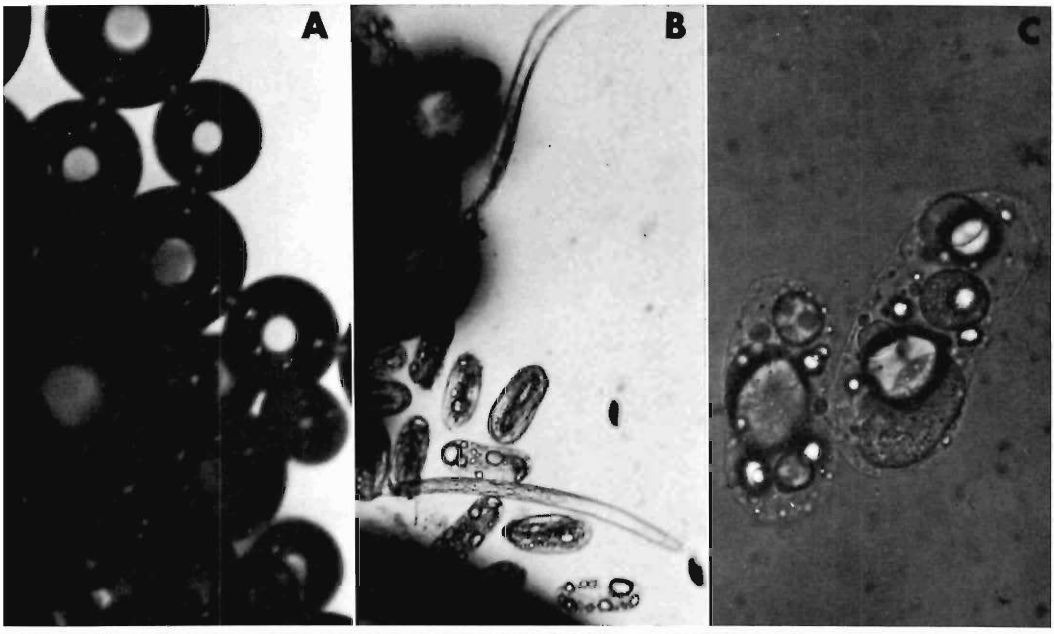


Figure 1. A *Heterodera glycines* cyst in undilute NaOCl. A, Bubbles evolving from cyst 3 min after immersion. B, eggs and larvae falling from ruptured cyst 3 min and 40 sec after immersion. C, Eggs 19 hr after immersion.

fiability in the weaker dilution series. This was the second most persistent living nematode tested.

Radopholus similis (male–female): Habit: Endo-Phytoparasitic

Dilution rate	Range Rupture Min	Sec	Min	Sec	Unidentifiable Min	Sec	Min	Sec
Undilute		30	1	15		40	2	30
1:5	1	10	1	40	1	10	2	20
1:10	2	40	8	55	4	00	9	15
1:15	6	40	256	15	11	55	256	15

In undilute and 1:5 dilution NaOCl a very fine cuticular layer peeled off followed by peeling away of the cuticle in layers. The body burst at various points. Males in undilute and 1:5 dilution deteriorated as females in 1:10. The one male checked deteriorated at a faster rate than the females.

Molting females in undilute and 1:5 dilution persisted more than 12 min before bursting or deteriorating. A molting male had its cast cuticle rapidly dissolved in a 1:5 solution. One female was unidentifiable in 3 min 25 sec in a 1:15 solution.

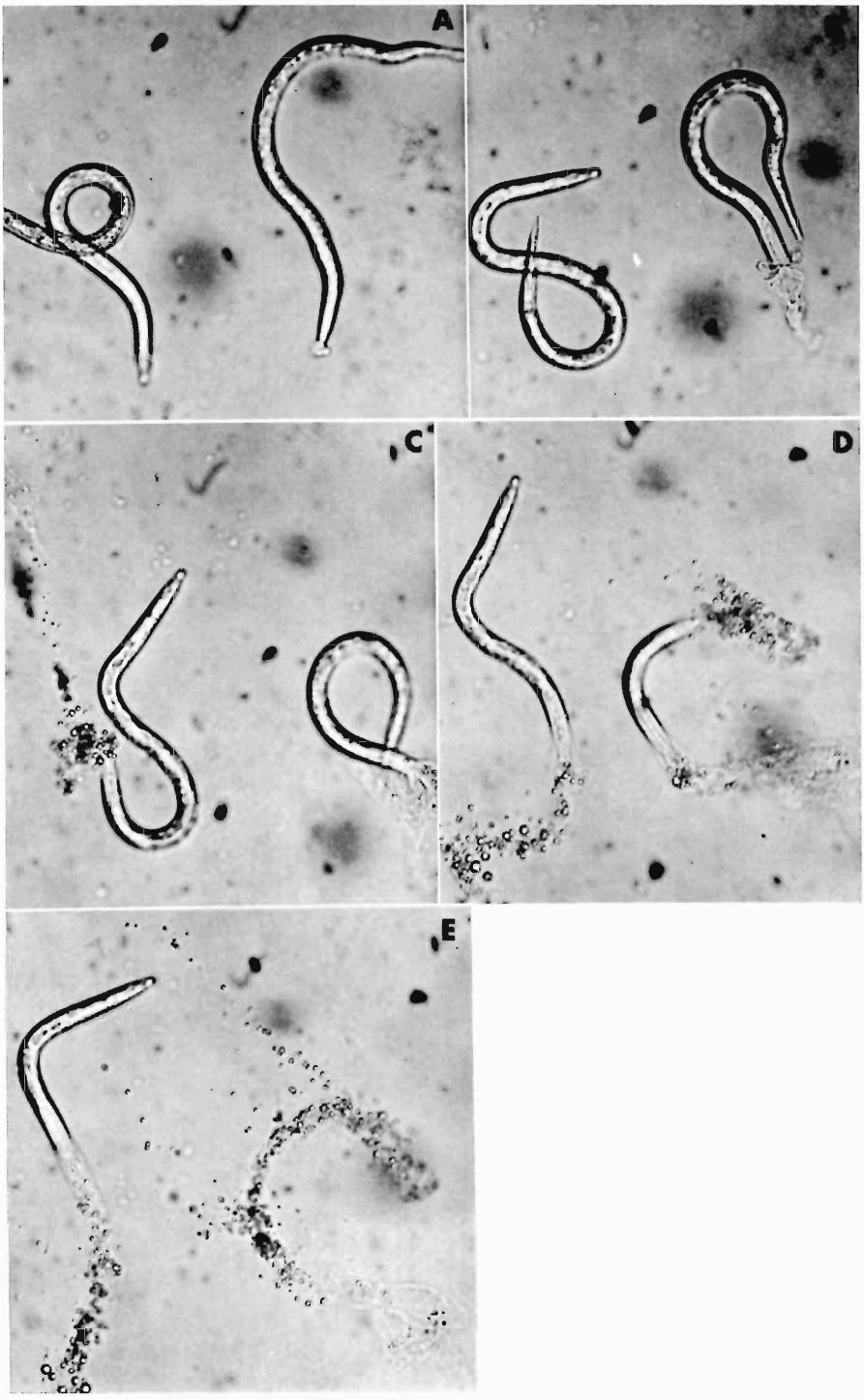
Pratylenchus sp. (females): Habit: Endo-Phytoparasitic

Dilution rate	Range Rupture Min	Sec	Min	Sec	Unidentifiable Min	Sec	Min	Sec
Undilute		20		35		45	1	05
1:5		35	1	25	1	15	2	25
1:10	1	55	4	50	3	20	8	45
1:15	5	45	22	50	12	30	22	50

Disintegration was gradual and uneventful. The body ruptured randomly at various locations.

Meloidogyne sp. (larvae and males): Habit: Endo-Phytoparasitic

Dilution rate	Range Rupture Min	Sec	Min	Sec	Unidentifiable Min	Sec	Min	Sec
Male								
Undilute		05		40		50	1	35
1:5	5	00	1	00	1	10	1	50
1:10	1	00	2	30	2	05	4	05
1:15	8	15	12	05	12	00	13	45
Larvae								
Undilute		25	1	05		45	1	20
1:5	1	40	4	55	2	45	5	30
1:10	3	00	4	35	3	45	4	55
1:15	3	00	27	20	3	30	27	20



Males in undilute and 1:5 solutions dissolved rapidly and uniformly and completely. Larvae also dissolved gradually and uneventfully (Fig. 2). Body rupturing was at random. Males succumbed rapidly and more dramatically than larvae.

Discussion

DECONTAMINATION DILUTION RATES: As an effective dip or drench to render unidentifiable nematodes contaminating laboratory equipment or substrates, a dilution of NaOCl at 1:5 exposed for 10 min would be most effective. Most nematodes were dissolved in less than 4 min (Table 1). Exceptions in this test include *Heterodera* cysts (unlikely as a contaminant), citrus nematodes, and some stages of ecdysis. Burrowing nematode, the chief nematode pest in Florida from a regulatory standpoint, was found easily dissolved at this dilution.

A 1:10 dilution for an exposed time of 10 min would also be effective for most nematodes. Most nematodes dissolve in less than 7 min. Cysts and citrus nematodes, however, would still be identifiable. The dilution rate of either 1:5 or 1:10 should prove a useful regulatory procedure in many laboratories.

The 1:15 rate would not be reasonably effective for dissolving nematodes since the exposure time would have to be excessively long.

It should be pointed out that all dilutions tested kill nematodes if a sufficient exposure occurs. However, kill alone was not an objective of this test.

HABIT: Free-living and ecto-phytoparasitic nematodes disintegrated easily and rapidly through the four series. Semi-endoparasitic nematodes (*Tylenchulus semipenetrans* larvae and *Heterodera* cysts) showed the strongest resistance to disintegration. Endo-phytoparasites proved quite susceptible to disintegration with a few specific exceptions.

SEX: Males of *Radopholus similis* and *Meloidogyne* sp. disintegrated more rapidly than the females or larvae. The reaction of larvae of two phytoparasitic genera was in sharp contrast; *Meloidogyne* sp. disintegrated easily while larvae of *Tylenchulus semipenetrans* persisted quite some time.

EXSHEATHMENT: NaOCl has been used to induce exsheathment (Lapage, 1933). In this study *Hemicycliophora* were ejected from the sheath forcibly, and sheath bonds to the body dissolved.

ECDYSIS: Molting specimens of *R. similis* persisted in undilute and 1:5 solutions 12 min prior to bursting or deteriorating. Other molting specimens of *R. similis* and *Helicotylenchus* deteriorated rapidly. In both cases the molted skin dissolved quickly. Indications are that some phases in the molting process are resistant to adverse chemical conditions while other periods are not.

MORPHOLOGY: Well-sclerotized structures such as spear, cephalic framework, and spicules persist for several minutes following body deterioration. Such structures are in an excellant state for study when the body has dissolved away. One *Hoplolaimus* sp. enface was made easily from a free cephalic framework. Definition of these structures are the best seen by the author. To preserve such structures water was added to stop the NaOCl action. To study cuticular layers the specimen was transferred to water as soon as the cuticle started peeling or layering.

BODY WALL: Particular attention was paid to what area of the body wall ruptured to determine if a particular part of the body wall structure is subject to rupturing indicating a weakness. No consistency occurred within genera or across the range of genera. Bursting of the body wall was random throughout its length. Body apertures rarely acted as a focal point for bursting. Within the random bursting, extrusion from the head occurred numerous times with several genera.

Literature Cited

Briggs, M. P. 1946. Culture methods for a free-living soil nematode. M.A. thesis, Stanford University.

←

Figure 2. Two *Meloidogyne* larvae placed in a 1:5 dilution of NaOCl. A, After 2 min and 10 sec, left larvae ruptures at head; B, After 3 min and 40 sec, larvae on right is still moving; C, In 4 min and 15 sec larvae on right ruptures; D, After 6 min and 15 sec both larvae are disintegrating; E, After 8 min and 5 sec the larvae on the right is disintegrated.

Chitwood, B. G. 1938. Further studies on nemic skeletoids and their significance in the chemical control of nemic pests. Proc. Helm. Soc. Wash. 5: 68–75.

Esser, R. P. 1963. Nematode interactions in plates of non-sterile water agar. Soil and Crop Sci. Soc., Fla. Proc. 23: 121–138.

Feder, W. A., and J. Feldmesser. 1955. Progress report on studies on the reproduction of the burrowing nematode, *Radopholus similis* (Cobb) Thorne, on citrus seedlings growing in Petri dishes. Plant Dis. Rept. 39: 395–396.

Guevara Pozo, D. 1953. Óbtención de huevos y larvas de áscaris en forma aséptica. Revista Ibérica de Parasit. 13: 333– 338.

Hirschmann, H. 1959. Histological studies on the anterior region of *Heterodera glycines* and *Hoplolaimus tylenchiformis* (Nematoda:Tylenchida). Proc. Helm. Soc. Wash. 26: 73–90.

Lapage, G. 1933. Cultivation of parasitic nematodes. Nature. London 131: 583–584.

Tyler, Jocelyn. 1938. Egg output of the root-knot nematode. Proc. Helm. Soc. Wash. 5: 49–54.

Development of Gametocytes and Oocysts of *Eimeria magna* from Rabbits in Cell Culture[1]

CLARENCE A. SPEER AND DATUS M. HAMMOND
Department of Zoology, Utah State University, Logan 84321

ABSTRACT: Merozoites of *Eimeria magna* were obtained from mucosal scrapings taken from a rabbit inoculated 5¼ days earlier. These merozoites were inoculated into cultures of Madin–Darby bovine kidney cells, which were then examined with interference-contrast or phase-contrast microscopy at intervals of 1 to 12 hr for 80 hr. Merozoites entered cells and most underwent development into gamonts and oocysts; some formed schizonts. From 12 to 60 hr, young macrogametes gradually enlarged; during this time, the plastic granules increased in size and number. Mature macrogametes were 28.5 by 21 μ. From 12 to 72 hr, microgamonts increased in size, nuclear divisions occurred and, in some, invaginations at the margin of the parasite were present. At 72 and 80 hr, mature microgamonts were 32 μ in diameter and had hundreds of microgametes. Extracellular microgametes exhibited slight motility after exposure to a 0.25% sodium taurocholate or 2.0% bovine bile solution. Fertilization was not observed. Mature oocysts were first seen at 72 hr after inoculation of merozoites.

Development of macro- and microgamonts of an eimerian species in cell culture has not yet been reported for any species occurring in mammals. The endogenous stages of *E. magna* in rabbits have been described by Rutherford (1943) and Cheissin (1960), and the in vitro development of first- and second-generation schizonts of this species has been reported by Speer and Hammond (1971). The development of merozoites to mature gametocytes and oocysts of *E. magna* from rabbits in cell culture is described herein.

Materials and Methods

Monolayer cell cultures of Madin–Darby bovine kidney (MDBK) cells (255th serial passage) were used to study the in vitro development of *Eimeria magna*. The methods of Fayer and Hammond (1967) were used to obtain and maintain the monolayer cell cultures. After 24 hr of incubation at 37 C, the monolayers of MDBK cells were inoculated with merozoites obtained by scraping the mucosa of the lower ⅔ of the small intestine of a rabbit inoculated 5¼ days earlier with approximately 200,000 oocysts of *E. magna*. The mucosal scrapings were gently stirred with a glass rod, and the mixture was then rinsed in saline A containing 5,000 μg dihydrostreptomycin and 5,000 units penicillin G/ml. The suspension was centrifuged at 200g for 1 min and the supernatant, containing the merozoites, was removed. The merozoites were then resuspended in minimal essential medium (MEM) with Eagle's balanced salt solution

[1] This investigation was supported by PHS Research Grant No. AI-07488-05 from the National Institute of Allergy and Infectious Diseases. Published as Journal Paper No. 1199, Utah Agricultural Experiment Station.