THE TOXOPLASMA GONDII OOCYST FROM CAT FECES*

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Toxoplasma infection is common in man and animals, yet for 60 years the life cycle of *Toxoplasma gondii* remained unknown. Recently a new form of *Toxoplasma* was found in the feces of cats that had eaten *Toxoplasma*-infected mice (for review of earlier work see [1]). This fecal form is biologically different from the known stages of *Toxoplasma*. While searching the feces of cats for a morphological equivalent of *Toxoplasma*, several candidate forms such as fungi, cysts of flagellates, and coccidian oocysts resembling those of *Isospora felis*, *I. rivolta*, and *I. bigemina* were found. Of these only oocysts resembling *I. bigemina* were constantly and quantitatively associated with fecal *Toxoplasma* infectivity. We will describe and characterize these oocysts and show by a series of mutually independent determinations that they should be regarded as oocysts of *Toxoplasma gondii*. Some of these findings were briefly reported (2).

DEFINITION OF TERMS

Trophozoites refer to intracellular and free forms of *Toxoplasma* which are actively proliferating in the tissues of acutely infected animals (Fig. 1). Free trophozoites are quickly digested in solutions of pepsin at pH 1.3.

Cyst refers to an accumulation of *Toxoplasma* (merozoites) characteristically occurring in the brain and muscle of chronically infected animals (Fig. 2). Cysts are surrounded by an elastic argyrophilic and periodic acid Schiff positive wall and contain much stored glycogen. The cyst wall is destroyed immediately on exposure to pepsin but the released merozoites survive in it for some time.

Oocyst is used in the coccidian sense. When used alone we refer specifically to the oocysts that resemble *Isospora bigemina* and which are excreted in feces of cats fed *Toxoplasma* (Figs. 3–9).

Although the terms cyst and oocyst may appear somewhat confusing, we felt it was desirable to retain these terms used for many years, until a better terminology can perhaps be devised after all of the developmental stages of *Toxoplasma* have been studied.

Infectivity refers to *Toxoplasma* infectivity of cat feces for mice, and is expressed also as minimal infective dose for 50% of inoculated mice (MID_{50}).[1]

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[1] *Abbreviations used in this paper:* MID_{50}, minimal infective dose for 50% of inoculated mice; SPF, specific pathogen free.

Materials and Methods

Adult cats were infected by feeding them tissues of mice with acute or chronic toxoplasmosis (*Toxoplasma* strain M-7741), or by the administration of infectious cat feces as previously reported (1). To exclude the possibility of activating a latent coccidial infection, 1–2 day old kittens, born from oocyst-free mothers, were fed *Toxoplasma* cysts by stomach tube. Control kittens and those fed *Toxoplasma* cysts were caged with their mothers in a room not previously occupied by cats. They were cared for by us personally to minimize contact with the Medical Center's main cat colony. Food consisted of Purina cat chow and canned dog food.

At autopsy, the intestine of each kitten or adult cat was divided in 6–10 segments. Each segment was flushed with ice-cold normal saline and the washings were collected for visual examination and for isolation of *Toxoplasma* in mice. Impression smears from each segment were fixed in acetone, in 5% acetic acid in 95% ethanol for fluorescent antibody staining, or in methyl alcohol for staining with Giemsa. Washed gut segments were flushed in ice cold fixative; they were preserved together with other internal organs in acetic alcohol for fluorescent antibody studies, and in Zenker-formol or 10% formalin for routine histological study.

Feces of cats were collected daily and floated in sucrose solution of 1.15 specific gravity containing 0.8% phenol as preservative. Washed "fecal floats" were preserved in 1 or 2% sulfuric acid or 2.5% potassium dichromate solution and aerated on a shaker at room temperature (23°–29°C) for 8 hr daily. For comparisons with fecal infectivity, oocyst counts were made of a 1/2500 ml of fecal float in a hemocytometer. 10-fold dilutions of fecal floats were fed by stomach tube or were injected intraperitoneally into groups of 2–6 mice. Infectivity was determined by the finding of *Toxoplasma* in impression smears or histologic sections, or by the development of dye-test antibodies at 12 days or longer after administering the inoculum.

To study oocyst sporulation, and when studying newborn kittens, fecal samples were collected from the rectum of infected cats. Oocysts were examined at intervals of 4–6 hr for the development of internal structures. Unless otherwise stated, development was studied at room temperature.

To obtain free sporozoites, sporulated oocysts were treated with 6% sodium hypochlorite solution (undiluted Purex) for ½ hr in an ice bath, washed with water, and then crushed between a cover glass and a slide which was coated with inactivated serum free of toxoplasmic antibody. Dried smears were fixed with methyl alcohol to be stained with Giemsa, or with acetone to be used for the fluorescent antibody test. Free sporozoites were also obtained by vigorously shaking Purex-treated oocysts with alundum (mesh No. 60) to release sporocysts and then treating the sporocysts with an excystation fluid consisting of 0.5% trypsin and 5% dog or cat bile in Melnick's A medium with 0.5% lactalbumin hydrolysate (pH 7.5) for ½–2 hr at 37°C.

For preparation of paraffin blocks of oocysts, fecal floats containing oocysts were mixed with 10% normal cat serum or 10% egg albumin and centrifuged to form a pellet which was fixed in acetic alcohol for 24 hr. Serial sections were cut at 5 μ.

For a comparison of oocyst filtrability and *Toxoplasma* infectivity, several filter systems were used. U.S. standard sieves were used down to 37 μ. The smallest pore size (25 μ) wire mesh sieve used was obtained from Baruch Instruments Corp., Ossining, N. Y. Perforated nickel foils with rated pore sizes of 10 and 15 μ were obtained from Perforated Products, Inc., Brookline, Mass. Columns of uniform spherical particles provide a filter bed that varies proportionally with the particle size. The interstices between uniform spherical particles were computed to be 15.4% of the diameter of the particles. "Micules" (spherical copolymer particles) were obtained from Sondell Scientific, Palo Alto, Calif. Glass beads were obtained from Minnesota Mining & Manufacturing Co., St. Paul, Minn. Mitex and Duralon filter discs were purchased from Millipore Filter Corp., Bedford, Mass., and Nucleopore filter discs were obtained from General Electric, Irradiation Processing Operations, Pleasanton, Calif.

Density characteristics of oocysts and *Toxoplasma* infectivity were compared by layering in-

fective cat feces on zonal and linear sucrose gradients made from 0.1 to 2 M sucrose solutions by methods similar to those described by Vetterling (3). After centrifugation, oocysts were counted in each of 10–15 fractions and infectivity was determined by mouse inoculation. A relatively clean and lipid-free fecal suspension was used to obtain reproducible results.

For preliminary determination of density range for oocysts and *Toxoplasma* infectivity, preserved fecal suspensions were filtered through 88, 44, and 37 μ wire sieves, and washed twice with 20 volumes of water by centrifugation. Washed feces were suspended in detergent 1% Tween 80 (Atlas Chemical Industries, Inc., Wilmington, Del.) in saline and kept on a shaker overnight. After washing off the detergent, feces were centrifuged in 2 M sucrose solution at 3000 rpm (2000 *g*) for 15 min and oocysts were collected from the supernatant fluid. Many fecal particles remained with the oocysts. Some of these fecal particles were removed by passing oocysts through 5 cm high columns of glass beads 420, 200, 100, and 60 μ in diameter. Oocysts were retained by 60 μ glass beads from which they were recovered by differential sedimentation. To remove lipid material, oocyst suspensions were treated with anesthetic ether and 95% ethanol (50:50) for 30 min, centrifuged at top speed for 10 min in a clinical centrifuge, and the supernatant fluid discarded. Portions of such treated oocyst preparation were centrifuged at 40,000 rpm (149,000 *g*) in a refrigerated centrifuge for 5 hr in separate concentrations of 0.3–2 M sucrose solutions. In each sucrose concentration oocysts were counted in the supernatant and in the sediment. Oocysts and infectivity were in the sediment in 0.3–0.5 M sucrose solutions. 80–95% of sporulated oocysts and infectivity were in the supernatant of 0.7–1.0 M sucrose solution. Therefore, for precise determination of density, oocysts were cleaned preliminarily by floating infectious cat feces in 1.15 M sucrose solution. Linear sucrose gradients were made by mixing equal volumes of 0.5 and 1.15 M sucrose solutions. Oocysts were either layered on the top layer of the gradient or mixed in the sucrose solutions before making the gradient. The tubes were centrifuged at 20,000 rpm (40,000 *g*, 4°C, Beckman Centrifuge, Beckman Instrument, Inc., Fullerton, Calif.) for 2–15 hr. By using a tightly fitting cap with two needles (inlet and outlet), 2 M sucrose solution was pumped to the bottom of the tube through the inlet needle and 10–15 fractions were collected from the top via the outlet needle.

For a comparison of the mobility of oocysts and infectivity in an electric field, fecal samples were fed at a rate of 20–30 μl/min into a continuous particle electrophoresis apparatus (Beckman, model No. 5, voltage gradient 75 v/cm, cell voltage 590 v/DC, cell current 16–24 ma). After passing the current, 48 fractions were collected and examined visually for oocysts and administered to mice for infectivity. Fractions 1–15 were positively charged, fraction 17 was neutral, and fractions 18–48 were negatively charged. Temperature was 4–5°C.

Antisera against *Toxoplasma* were prepared in mice either by feeding oocysts or by injecting *Toxoplasma* cysts. *Toxoplasma* antibody titers were determined by the Sabin and Feldman dye test (4). For controls, antisera were absorbed with trophozoites of the standard RH strain of *Toxoplasma*. Fluorescein isothiocyanate-tagged anti-mouse globulin was obtained from the National Instrument Laboratories, Inc., Rockville, Md. Batches of swine anti-host globulin free of *Toxoplasma* antibodies were selected.

For the indirect-fluorescent antibody test smears were prepared from crushed oocysts, infected cat intestinal epithelium, and from the peritoneal exudate of mice infected 2 days previously with the standard RH strain of *Toxoplasma*. Smears were fixed with acetic alcohol or acetone and stored at −20°C. Deparaffinized sections or smears were treated with anti-*Toxoplasma* sera for 1 hr at 37° and then overnight at 4°C. After washing, slides were treated with anti-mouse globulin for 60 min at 37°C. The slides were washed, air dried, and mounted in 50% glycerine (pH 7.6).

Additional details, maintenance of *Toxoplasma* strain used, diagnosis of *Toxoplasma* infection in cats and in mice, and details of the dye test used are as described previously (1).

Line drawings are composites of many oocysts. All measurements are in microns and are made from potassium dichromate-preserved oocysts. Mean values are given with the range in parentheses.

RESULTS

Excretion of Oocysts and Infectivity in Feces of Cats Fed Toxoplasma Cysts, Trophozoites, and Infectious Cat Feces (Table I).—The excretion of oocysts and of *Toxoplasma* infectivity began simultaneously in cats, 3–5 days after feeding cysts, 7–10 days after feeding trophozoites, and 20–24 days after feeding infected cat feces. The frequency of excreting fecal forms of *Toxoplasma*, the serology and the fate of the cats have been reported earlier (1). Oocysts were not found in the feces of any of 50 cats prior to feeding *Toxoplasma.*

Description of the Oocysts.—In freshly passed oocysts, the sporont was granular and completely filled the oocyst (Figs. 3, 4, and 15). After 6–9 hr the sporont contracted (Figs. 5 and 16) and two sporoblasts were seen after 9–12 hr without a change in the oocyst shape (Figs. 6 and 17). The sporoblasts then elongated to form two sporocysts at each end of which light areas interpreted as nuclei were seen (Figs. 7 and 18). Four sporozoites appeared in each sporocyst between 21 and 28 hr; maximum development occurred after 48 hr of incubation at room temperature. The oocysts had a light greenish tinge.

TABLE I

Appearance of Oocysts and Toxoplasma Infectivity in Feces of Dye-Test Negative Cats Fed Toxoplasma Cysts, Trophozoites, and Infectious Cat Feces

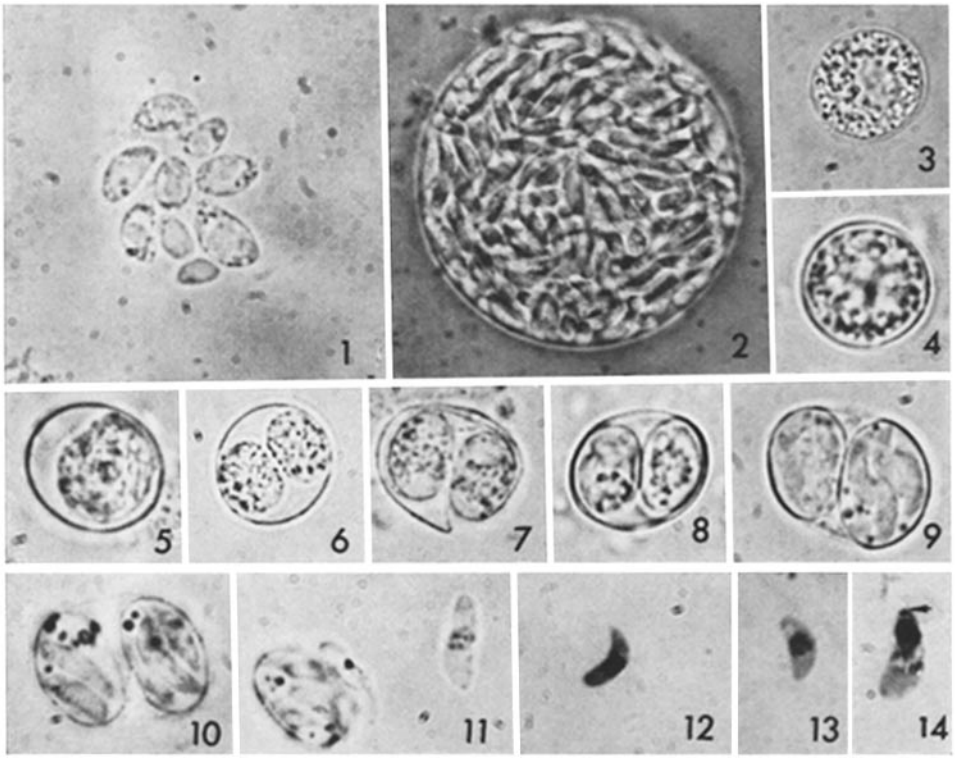
	Toxoplasma stage fed		
	Cysts	Trophozoites	Infected feces (oocysts)
Oocysts appearance (Days)	3–5	7–9	20–24
Infectivity appearance (Days)	3–5	7	20–24
No. of cats with infectious feces/No. of cats fed	23/24	4/9	8/17

Unsporulated oocysts (Figs. 3, 4, and 15) were subspherical to spherical. 100 oocysts measured 10 × 12 (9–11 × 11–13); their length–width ratio was 1.15 (1.0–1.45). Oocyst walls were colorless, smooth, and about 0.5 μ thick. Micropyle and polar granule were absent.

Sporulated oocysts (Figs. 8 and 19) were subspherical to ellipsoidal. 100 oocysts measured 11 × 12.5 (10–11 × 11–14); their length–width ratio was 1.13 (1.0–1.50). Sporulated oocysts appear to have two smooth layers; the outer layer could be removed by treating oocysts with 6% sodium hypochlorite solution for ½ hr (Figs. 9 and 10). An oocyst residuum was absent.

Each sporulated oocyst contained two ellipsoidal sporocysts without a Stieda body (Figs. 10 and 20). 100 free sporocysts measured 6 × 8.5 (5.0–6.5 × 8.0–9.5); their length–width ratio was 1.41 (1.25–1.70). The sporocyst residuum consisted either of compact granules lying at one end of the sporocyst or of a few scattered granules; both types of sporocyst residua were occasionally seen together inside the same oocyst (Figs. 8 and 19).

There were four sporozoites in each sporocyst as determined by direct inspection, by counts of nuclei in stained sections, and by examination during excystation. They were elongated and curved within each sporocyst (Figs. 10, 11, and 20). They measured approximately 2 × 8 when free. After staining with Giemsa, a nucleus was seen lying towards the middle of each sporozoite (Figs. 12, 13, and 21); occasionally a conoid could be recognized at the anterior end (Fig. 14). No other structures were seen in unstained or stained sporozoites.



FIGS 1–14. Photomicrographs of different stages of *Toxoplasma*. × 1600. Figs 1–11 are unstained fresh preparations, Figs 12–14 are stained with Giemsa.

FIG. 1. Trophozoites from peritoneal exudate of a mouse infected with RH strain.

FIG. 2. Cyst from a mouse brain infected with M-7741 stran. The cyst wall enclosing the numerous merozoites is clearly shown.

FIGS. 3 and 4. Oocysts, unsporulated in freshly passed cat feces. The oocyst in Fig. 3 is slightly flattened.

FIG. 5. Oocyst with contracted sporont after 9 hr aerobic development at room temperature.

FIG. 6. Oocyst with two sporoblasts after 12 hr aerobic development at room temperature.

FIG. 7. Oocyst with two sporocysts after 18 hr aerobic development at room temperature. Light areas interpreted as nuclei are seen at both ends of sporocysts. The oocyst wall has ruptured during preparation of the oocyst mount.

FIG. 8. Oocyst sporulated after 24 hr aerobic development at room temperature. Sporocyst residua are in focus, appearing as only a few granules in one sporocyst and as a ball in the other.

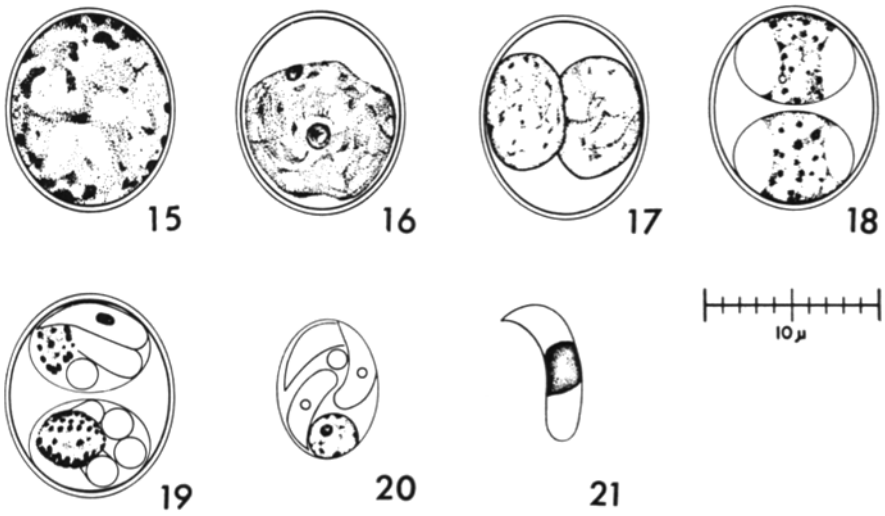
FIGS. 9 and 10. Oocysts sporulated 30 min after treatment with 6% sodium hypochlorite solution. Note the thinness of the wall. The outer wall has been dissolved. Fig. 9. All four sporozoites are in focus in one sporocyst. Fig. 10. Sporocysts showing the arrangement of sporozoites.

FIG. 11. Free sporozoite released from sporocyst by pressure on cover slip.

FIGS. 12–14. Sporozoite stained with Giemsa. Representative variation in shape, nuclear position, and staining at the tip are shown. Fig. 12. Elongated form with nucleus in the middle.

Fig. 13. Crescentic form with nucleus in the middle. Fig. 14. Nucleus and a conoid at the anterior end.

Excretion of Oocysts and Infectivity by 1–2 Day-Old Kittens Fed Toxoplasma Cysts (*Table II*).—Oocysts and infectivity (after incubation) were found in the gut washings of all 8 kittens killed between 3 and 9 days after being fed *Toxoplasma* cysts. They were absent in the gut washings of kittens killed 1 and 2 days after being fed cysts, and in the controls. Schizogonic and gametogonic stages (Figs. 22–28) were found in the gut of 10 out of 11 kittens fed *Toxoplasma* cysts but not in the uninfected litter-mate controls. The asexual cycle of *Toxoplasma* in the cat intestine comprises several generations of schizonts and will be described in detail elsewhere.



FIGS. 15–21. Line drawings of *Toxoplasma* oocysts, sporocyst, and sporozoite drawn to the same scale.

FIG. 15. Unsporulated oocyst with sporont occupying the entire inner mass.

FIG. 16. Unsporulated oocysts with contracted sporont.

FIG. 17. Oocyst with two sporoblasts.

FIG. 18. Oocyst with two sporocysts. Note nuclei at both ends.

FIG. 19. Sporulated oocyst with two sporocysts containing sporozoites. Note the variation in sporocyst residua.

FIG. 20. Sporocyst with sporozoites and a residual mass.

FIG. 21. Sporozoite with a nucleus.

Appearance, Disappearance, and Quantitative Comparison of Oocysts and Infectivity in Feces of Cats Fed Toxoplasma Cysts (*Table III*).—In order to exclude a chance association of oocysts and infectivity, fecal floats from separate cats, and on different days after they were fed *Toxoplasma* cysts, were titrated in mice, and oocyst counts were made independently. Oocysts and infectivity appeared and disappeared in the feces at about the same time (Table III). An additional 113 fecal samples from cats fed *Toxoplasma* were examined visually

and administered to mice for *Toxoplasma* infectivity. Some of these are presented with other experiments (Tables IV–VIII).

Comparison of Oocyst Numbers and Infectivity by Oral and Intraperitoneal Routes in Mice.—Fecal specimens were titrated by the oral route in mice and compared with oocyst numbers. Infectivity was found to be within 1 log of the oocyst numbers in 28%, 1 log lower than oocyst numbers in 54%, and 2 logs lower in 10% of 82 fecal samples. Thus, the infectivity titer was always lower

TABLE II

Excretion of Toxoplasma Oocysts in Feces of Young Kittens Fed Toxoplasma Cysts

Experiment No.	Kitten No.	Cysts fed(+) or control	Day killed after infection	Dye test kittens (autopsy)	Isolation of Toxoplasma*	Oocysts in feces	Fecal infectivity	Tissue stages gut‡
9§	1	+	1	<1:2	+	−	−	−
7§	1	+	2	1:16	+	−	−	+
11§	1	+	2	>1:128	+	−	−	+
	2	+	3	>1:128	+	+	+	+
	3	+	5	1:128	+	+	+	+
	4	control	6	1:64	−	−	−	−
3‖	1	+	5	1:32	+	+	+	+
	2	+	7	1:32	+	+	+	+
	3	+	8	1:8	+	+	+	+
4§	1	+	5	>1:128	+	+	+	+
	2	+	7	1:128	+	+	+	+
	3	+	9	>1:128	+	+	+	+
	4	control	9	1:128	−	−	−	−

* Liver, lung, spleen of kittens inoculated subcutaneously into mice.

‡ Schizogonic or gametogonic stages.

§ 1-day old kittens born of mothers with dye-test titer greater than 1:64.

‖ 2-day old kittens born of mothers with dye-test titer of 1:32.

than the counted oocysts when using the oral route in mice. Comparison of the same fecal sample by oral and by intraperitoneal routes showed that higher infective titers were obtained by the intraperitoneal titration. This suggested loss of oocysts with feeding. Comparison of oocysts fed to mice and oocysts found in their feces showed that approximately 10% of the inoculum passed through the mouse gut. A greater percentage of unsporulated than sporulated oocysts passed through the gut.

Effect of Chemicals on Oocyst Sporulation and Infectivity (Tables IV and V).—Oocyst sporulation and the development of *Toxoplasma* infectivity were affected to the same degree by different chemicals. Oocysts did not sporulate nor

did infectivity develop in 0.3% formalin, in 1% iodine in 20% ethanol, or in 1% ammonium hydroxide solution. In 1 or 2% sulfuric acid, or in 2.5% potassium dichromate solution 70–80% of the oocysts sporulated. In 20% ethanol or in tap water, only 46–36% of the oocysts sporulated.

The number of counted oocysts best correlated with the number of oocysts preserved in sulfuric acid titrated intraperitoneally (Table V). Dichromate staining of the oocyst wall could not be completely removed by washing with water, suggesting interference with the enteric enzymes necessary for oocyst digestion.

Effect of Temperature on Sporulation of Oocysts and Development of Infectivity (Tables VI, VII, and VIII).—When collected form the rectum of cats, oocysts were undeveloped and were not infective to mice. Oocyst sporulation and the development of *Toxoplasma* infectivity proceeded in parallel manner at room and at lower temperatures (Table VI). At 25°C oocyst sporulation occurred between 24 and 28 hr and infectivity also developed at nearly the same time. A 10-fold rise in the number of sporulated oocysts between 28 and 32 hr was associated with a similar rise in the infectivity titers. Maximum oocyst sporulation at 48 hr was associated with development of maximum infectivity titers. These results were consistent in a total of three experiments done with feces from different cats.

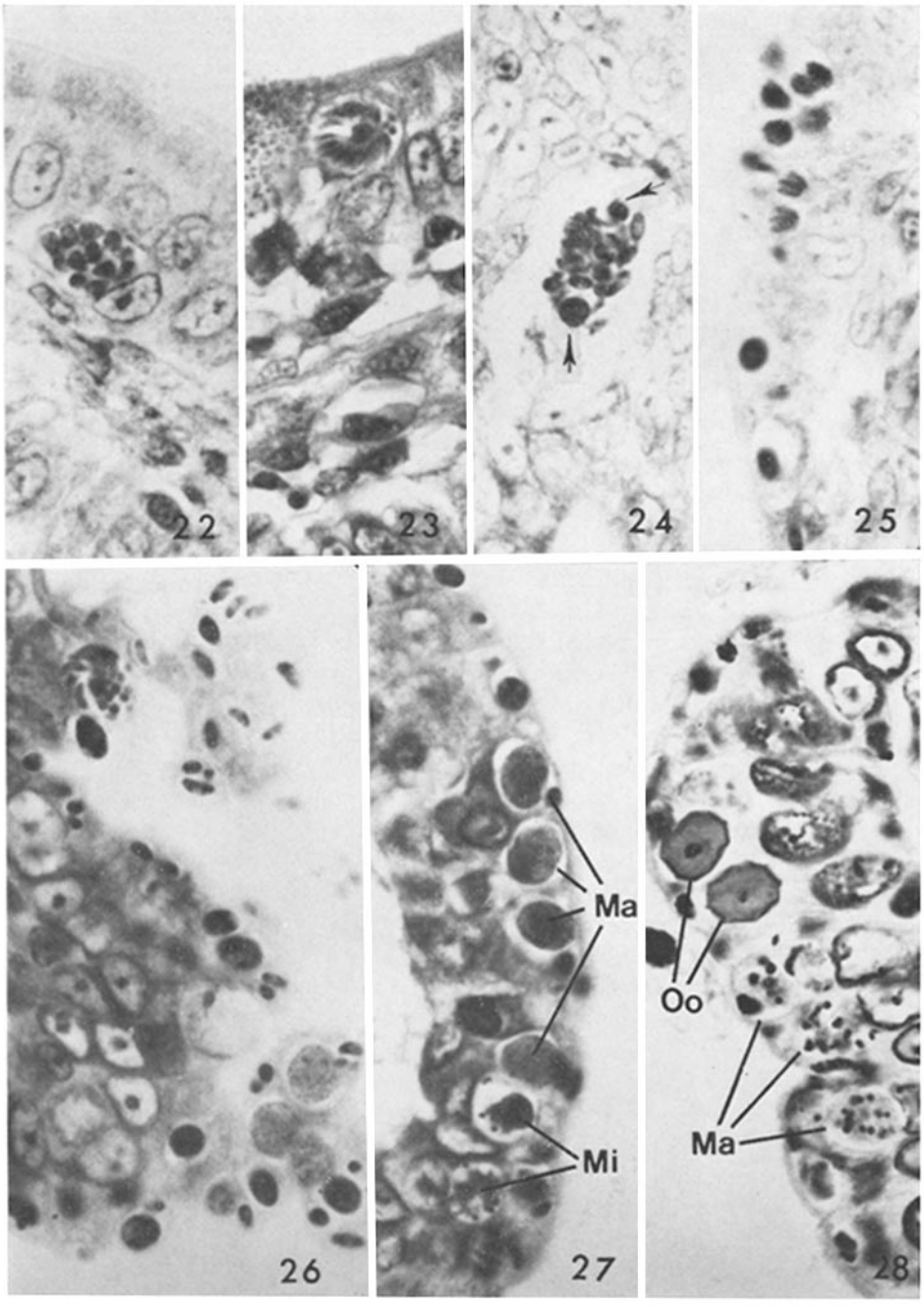
At 15°C, oocysts sporulated in 2–5 days and the infectivity also developed at the same time. A rise in the number of sporulated oocysts between 5 and 8 days was associated with a comparable rise in infectivity.

At 11°C oocyst sporulation was delayed until 21 days and the development of infectivity was similarly delayed. More importantly, the number of sporulated oocysts were comparable to infectivity titers at each incubation period. At 4°C neither oocysts nor infectivity developed.

Storage of feces at 37°C for 8 hr reduced the oocyst sporulation from 70 to 28%. Sporulation of oocysts and development of *Toxoplasma* infectivity were prevented by 24 hr exposure to a temperature of 37°C (Table VII). Arrest of sporogony occurred at progressively earlier stages with increasing exposure times. 1-hr exposures to temperatures of 40–45°C were not very harmful. But heating feces to 50°C for only 10 min prevented both oocyst sporulation and the development of *Toxoplasma* infectivity (Table VIII). Consistent findings were obtained with three fecal samples.

Effect of Aeration and Anaerobiosis on Oocyst Sporulation and Development of Infectivity (Table IX).—In aerated samples, oocysts sporulated and infectivity developed within 24 hr. Anaerobiosis affected both adversely. In one fecal sample incubated with bacteria in thioglycollate broth, oocysts and infectivity did not develop during 30 days. After the same sample was exposed to air for 5 days, oocysts sporulated and infectivity developed. However, only 4% of oocysts sporulated, compared with 50–65% of the controls.

Comparison of Oocyst Size and Filtrability of Infectivity (Table X).—It had



been shown previously that *Toxoplasma* infectivity passed through the smallest, 37 μ U.S. standard sieve (5). To bridge the gap between 37 μ to below the observed oocysts size (variability of 9–14 μ) several filter systems were employed.

Both oocysts and infectivity passed through a 25 μ wire mesh sieve and through 10 and 15 μ electrofoils, the pore ratings of which were confirmed with a slide micrometer. Oocysts and infectivity passed through a column of 115 μ Micules with interstices (effective pore size) of 18 μ. Very few oocysts and little infectivity passed through a column of 60 μ Micules with a pore rating of 9 μ, and none passed through a column of 50 μ Micules with an effective pore size of 8 μ, or through a column of 60 μ glass beads with an effective pore size of 9 μ. These findings coincided very closely with the observed smallest oocyst size of 9 μ. Oocysts and infectivity were also retained by 5 and 8 μ Nucleopore filters.

Variable results were obtained by using Duralon, Mitex, and sintered glass filters. Oocysts and infectivity were retained by 7 and 14 μ Duralon filters. They were also completely retained by a medium size sintered glass filter (Pyrex) with a pore rating of 10–15 μ. However, 5 and 10 μ Mitex filters permitted some oocysts and infectivity to pass.

Comparison of Density Gradient Fractions of Oocysts with Infectivity (Fig. 29).—In 21 sucrose gradients it was determined that the density range for oocyst and infectivity was between concentrations equivalent to 0.6 and 1.10M sucrose solution. When clean oocysts obtained by floating feces in 1.15M sucrose solution were layered on a continuous sucrose gradient, banding of oocysts and infectivity occurred between concentrations equivalent to 0.82 and 1.12M sucrose solution (specific gravity 1.104 and 1.140). The peak number of oocysts and highest titer of *Toxoplasma* infectivity were at 0.92M sucrose solution (specific gravity 1.11). This was confirmed by two additional determinations.

FIGS. 22–28. Photomicrographs of representative schizogonic and gametogonic stages of *Toxoplasma* in the small intestinal epithelium of kittens fed *Toxoplasma* cysts. × 1200.

FIG. 22. Postdivisional *Toxoplasma* in an epithelial cell which apparently lost its orientation. 36 hr after infection, Giemsa stain.

FIG. 23. Segmenting *Toxoplasma* schizont with eosinophilic residual body in the center of the group. 48 hr after infection, H & E stain.

FIG. 24. *Toxoplasma* schizont with bipolar organisms and two eosinophilic residual bodies (arrows). 48 hr after infection, Giemsa stain.

FIG. 25. *Toxoplasma* schizonts, immature and segmenting. 67 hr after infection, Giemsa stain.

FIG. 26. Bipolar merozoites, macrogametocytes, and developing schizonts and gametocytes. 8 days after infection, Giemsa stain.

FIG. 27. Two microgametocytes (Mi), four macrogametocytes (Ma), and a schizont (below). 8 days after infection, Giemsa stain.

FIG. 28. Two oocysts (Oo) in epithelium showing the thin oocyst wall and a central nucleus. Next to it are three macrogametocytes (Ma) which show prominently staining cytoplasmic granules. 8 days after infection, Wilder's ammoniacal silver impregnation.

Attempted Separation of Oocysts and Infectivity by a Continuous Particle Electrophoresis (Fig. 30).—When a floated fecal sample was run in continuous particle electrophoresis, oocysts and *Toxoplamsa* infectivity were confined to 11 of 40 fractions. Oocysts and infectivity had a broad peak between fractions 23 and 26.

When two runs were made from unfloated feces (filtered through 88, 44, and

TABLE III

Appearance, Disappearance, and Quantitative Comparison of Oocysts and Toxoplasma Infectivity in Feces of Cats Fed Toxoplasma Cysts

Experiment No.	Post feeding	Oocysts in feces (counted)	Infectivity titer*
	days		
	3	—	—
C.T. 8	4	—	—
−1628	5	$10^{5.21}$	$10^{5.0}$
	14	+	$10^{2.0}$
	15	—	$10^{1.0}$
	18	—	—
C.T. 8	3	—	—
−1639	5	$10^{5.72}$	$10^{6.0}$
	12	$10^{4.13}$	$10^{4.0}$
	2	—	—
C.T. 13	3	+ (unsporulated)	—
−1	4	$10^{6.59}$	$10^{5.6}$
	5	$10^{6.37}$	$10^{4.8}$
	3	—	
C.T. 23	4	+ (unsporulated)	—
−1	5	$10^{3.97}$	$10^{4.0}$‡
	7	$10^{5.0}$	$10^{4.69}$‡

* Fecal samples were preserved in 1% sulfuric acid for 7 days and titrated orally in mice.
‡ Intraperitoneal titrations.

37 μ U.S. Standard sieves), both oocysts and infectivity were distributed in 48 tubes. Sporulated oocysts were found between fractions 1 and 42 and were associated with infectivity; unsporulated oocysts occurred between fractions 43 and 48 and were not infective to mice.

Identification of Oocyst and its Tissue Stages with Anti-Toxoplasma Sera by Fluorescent Antibody Technique.—Oocyst and sporocyst walls, sporozoites in smears of crushed oocysts, and schizonts and female gametocytes in gut smears were stained with *Toxoplasma* sera; this staining could be abolished by adsorb-

ing the sera with the standard RH strain of *Toxoplasma*. In the paraffin sections, only oocyst and sporocyst walls were stained, although after staining them with Giemsa, sporozoites were found inside the sporocysts. Schizonts and male and female gametocytes as shown in figures 22–28 were not stained with fluorescent antibody in paraffin sections, although *Toxoplasma* trophozoites from mesen-

TABLE IV

*Comparison of Preservatives for Oocyst Sporulation and Development of Toxoplasma Infectivity**

Reagent	Total No. of oocysts	Oocyst stages			Total No. of sporulated oocysts	Infectivity (oral)
		Sporonts	Sporocysts	Sporozoites		
		%	%	%		
1% sulfuric acid‡	$10^{6.54}$	32	0	68	$10^{6.37}$	$10^{4.8}$
2% sulfuric acid‡	$10^{6.51}$	26	0	74	$10^{6.38}$	$10^{4.8}$
2.5% potassium dichromate‡	$10^{6.55}$	20	0	80	$10^{6.46}$	$10^{4.8}$
20% ethanol‡	$10^{6.62}$	42	16	42	$10^{6.25}$	$10^{3.8}$
Water (control)‡	$10^{6.49}$	36	28	36	$10^{6.04}$	$10^{4.2}$
0.1% formalin	$>10^{3.0}$	80	17	3	N.D.	positive
0.3% formalin	$>10^{3.0}$	80	20	0	0	negative
1.0% ammonium hydroxide	$>10^{3.0}$	100	0	0	0	negative
1% iodine in 20% ethanol	$>10^{3.0}$	100	0	0	0	negative

* Fecal floats were incubated for 7 days to a depth of 3 mm on a shaker at 23–24°C.
‡ Same fecal sample. N.D. = not determined.

TABLE V

Toxoplasma Infectivity of Sporulated Oocysts Preserved in Potassium Dichromate and Sulfuric Acid

Preservative	No. of titrations	Per cent infectivity by intraperitoneal route based on count of sporulated oocysts				
		<0.1	0.1–1.0	1–10.0	11–50	51–100
2.5% potassium dichromate	20	1 (5%)	3 (15%)	12 (60%)	3 (15%)	1 (5%)
1 or 2% sulfuric acid	20	0	0	7 (35%)	6 (30%)	7 (35%)

Per cent figures in parentheses indicate per cent of infectivity within each titration group.

teric lymph nodes of the same animal were stained. However, on smears schizonts, macrogametocytes, and oocysts were similarly stained.

Comparison of Toxoplasma Infectivity of Oocysts Before and After Excystation.—Sporulated oocysts contain eight sporozoites. If each sporozoite were an infective unit of *Toxoplasma*, the infective titer of excysted oocysts should be eight times higher than that of intact oocysts. To test this, a sample of oocysts was treated with 6% sodium hypochlorite for 30 min. After washing, the oocysts were equally divided into two samples. One was mixed with excystation fluid

TABLE VI

Effect of Temperature on Oocyst Sporulation and Development of Toxoplasma Infectivity

Temperature	Time exposed	Oocyst Stages				Total No. of sporulated oocysts	Infectivity (intraperitoneal)
		Sporonts	Sporoblasts	Sporocysts	Sporozoites		
°C		%	%	%	%		
37*	5 days	100	0	0	0	0	0
25‡	3 hr	100	0	0	0	0	0
(room temp)	19	30	24	46	0	0	0
	24	24	10	66	0	0	$10^{1.0}$
	28	22	10	67	1	$10^{2.97}$	$10^{2.69}$
	32	24	6	60	10	$10^{3.97}$	$10^{4.0}$
	48	28	0	7	65	$10^{4.78}$	$10^{4.06}$
	2 days	100	0	0	0	N.D.	0
15*	5	36		63	1	N.D.	10^{0}
	8	40		20	40	N.D.	10^{2}
	2 days	100	0	0		0	0
11*	9	23	22	55	0	0	0
	14	16	12	72	0	0	0
	21	16	4	40	40	$10^{4.72}$	$10^{4.0}$
	28	15	0	0	85	$10^{5.06}$	$10^{4.0}$
4*	60 days	100	0	0	0	0	0

* Samples were incubated in 2.5% potassium dichromate to a depth of 3–4 mm.

‡ Samples were incubated in 2% sulfuric acid.

N.D. = not determined.

TABLE VII

*Effects of Exposures of Feces to a Temperature of 37°C on Oocyst Sporulation**

Time exposed	Oocyst stages				Infectivity (oral)
	Sporonts	Sporoblasts	Sporocysts	Sporozoites	
	%	%	%	%	
(control) 16 days	30	0	0	70	+
4 hr	34	0	4	62	N.D.
6	50	0	10	40	N.D.
8	56	0	18	28	N.D.
12	58	0	30	12	N.D.
16	74	9	14	3	N.D.
20	88	6	5	1	N.D.
24	97	3	0	0	—

* Unsporulated oocysts preserved in 2.5% potassium dichromate solution were incubated in capped bottles to a depth of 3–4 mm at 37°C for 4–24 hr. Each group was then kept at room temperature and the degree of sporulation was determined after 16 days.

N.D. = not done.

and kept on a shaker at 37°C. The other sample was mixed in Melnick's A medium only and incubated at 37°C. After 2 hr, both samples were removed to an ice bath and twofold titrations were made in Melnick's A medium. Samples of each dilution were injected into mice.

TABLE VIII

Effect of Heating Feces on Oocyst Sporulation and on the Development of Toxoplasma Infectivity

Temperature	Time exposed	Oocyst stages			Total No. of sporulated oocysts	Infectivity* (intraperioneal)
		Sporonts	Sporocysts	Sporozoites		
°C		%	%	%		
22–25	1 hr	28	0	72	$10^{5.06}$	$10^{4.06}$
35	1	30	0	70	$10^{4.97}$	$10^{3.69}$
40	1	30	0	70	$10^{5.0}$	$10^{4.69}$
45	1	30	11	59	$10^{4.94}$	$10^{4.06}$
50	10 min	100	0	0	0	0
	20	100	0	0	0	0

* Fecal samples were preserved in 2% sulfuric acid and examined after 7 days.

TABLE IX

*Effect of Oxygenation on Oocyst Sporulation and Development of Toxoplasma Infectivity**

Aerobic‡					Anaerobic§				
Time	Oocyst Stages			Infectivity (oral)	Time	Oocyst Stages			Infectivity (oral)
	Sporonts	Sporocysts	Sporozoites			Sporonts	Sporocysts	Sporozoites	
hr	%	%	%		*days*	%	%	%	
3	100	0	0	—	2	88	12	0	—
24	30	57	13	+	3	52	48	0	—
28	22	48	30	$10^{1.0}$					—
32	24	26	50	$10^{3.0}$					—
48	24	11	65	$10^{3.0}$	30	80	20	0	—
168	32	18	50	$10^{3.0}$	30 anaerobic + 5 days aerobic‖	66	30	4	+

* Same fecal suspension was used for both groups, room temperature.

‡ Oocysts preserved in 2.5% potassium dichromate solution to a depth of 5 mm on a shaker.

§ Unpreserved oocysts were incubated at room temperature in thioglycollate broth to a depth of 8 cm.

‖ After 30 days of anaerobic incubation the fecal sample was mixed with 2% sulfuric (50:50) and poured into a Petri dish to a depth of 5 mm.

Titration of a sample of 10,000 sporulated oocysts indicated an infectivity of 12,800 infectious doses before excystation, and of 51,200 after excystation. Thus there was a fourfold rise in infectivity titer after excystation of oocysts.

Failure of Dogs to Excrete Isosporan Oocysts or Toxoplasma Infectivity (Table

TABLE X

Filtrability of Oocysts and Toxoplasma Infectivity

Filter system	Pore rating	No. of oocysts in 10% of filtrate	Infectivity to mice*	
			Day of death	Serology
	μ			
Electrofoil	15	79	9, 9	
Electrofoil	10	77	9, 9	
115 μ Micules	18	40	7, 12	
60 μ Micules	9	3	12, 12	
50 μ Micules	8	0	Surv.	Neg.
60 μ glass bead	9	0	Surv.	Neg.
Mitex	10	4	12, 14	
Mitex	5	3	Surv.	Pos.
Duralon	14	0	Surv.	Neg.
Duralon	7	2‡	Surv.	Neg.
Nucleopore	8	1‡	Surv.	Neg.
Nucleopore	5	0	Surv.	Neg.
Control (unfiltered)		214	8, 8	

* 40% of the filtrate was fed to each of two mice. An earlier death reflects a higher number of organisms inoculated than does a later death or seroconversion.

‡ Unsporulated, collapsed.

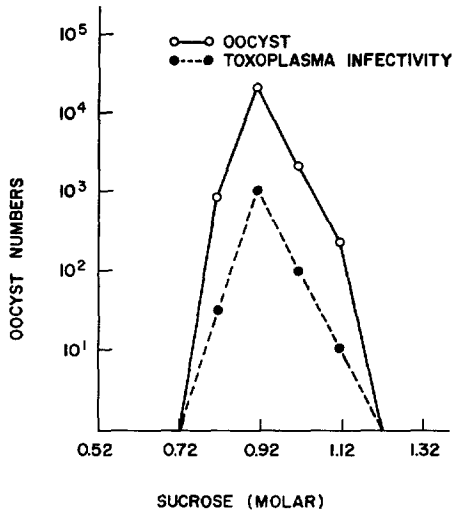


FIG. 29. Distribution of oocysts and *Toxoplasma* infectivity in a linear sucrose gradient. Oocysts were preserved in 2.5% potassium dichromate solution. Gradient was centrifuged at 40,000 *g* for 2 hr.

XI).—Oocysts resembling *Isospora bigemina* previously described in this study have been found in feces of both dogs and cats (6). It was therefore of interest to us to find whether dogs would excrete these oocysts after being fed *Toxoplasma.*

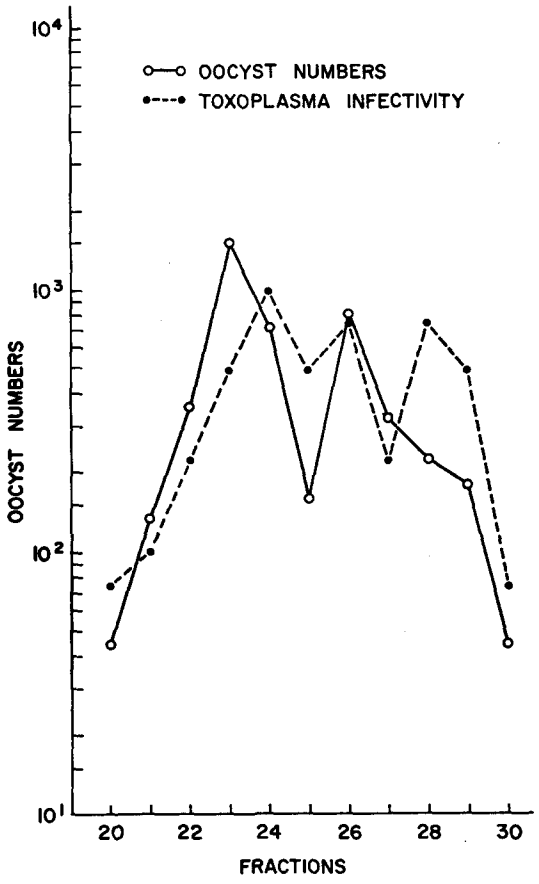


FIG. 30. Distribution of oocysts and infectivity by continuous particle electrophoresis (CPE). The oocysts were preserved in 1% sulfuric acid. The CPE instrument settings were: flow rate 25 ml/min, sample feed 30 μl/min, voltage 75 volts/cm, amperage 17 ma, and total voltage of 600 volts. The buffer was Tris buffer, pH 9.2, with a conductivity of 41 μ reciprocal ohms. Temperature 4–5°C.

None of 10 dog pups fed *Toxoplasma* cysts or oocysts excreted oocysts or *Toxoplasma* infectivity in their feces. All but two dogs became infected with *Toxoplasma* as shown by their development of *Toxoplasma* antibodies or by the isolation of *Toxoplasma* from their tissues.

To find whether other cat coccidia could be transmitted to dogs, six dog pups from two litters were fed *Isospora felis* and three dog pups were fed *Isospora*

rivolta oocysts from cat feces. None of these dogs excreted *I. felis* or *I. rivolta* in their feces although three of them were examined for 30 days. The two mother dogs who nursed these young dogs were free of oocysts before and after feeding the oocysts to their pups.

To prove that at least three dogs (Nos. 9–11) were not immune to *Isospora* of dog origin, they were fed 10^4–10^5 oocysts of *I. canis* and *I. rivolta* 30 days after they had been fed the oocysts of cat origin. One dog (No. 9) died prematurely on the 4th day but the other two excreted oocysts of *I. rivolta* (from day 8) and *I. canis* (starting on day 11).

TABLE XI

Failure to Excrete Oocysts by Dogs Fed Toxoplasma Cysts and Oocysts

Experiment No.	Dog No.	Age of dog	Inoculum	Day killed or died after feeding	Dye test (dog)		Isolation of *Toxoplasma*	Oocysts in feces	Fecal infectivity
					Pre-feeding	Post-feeding			
		days							
D.T. 1	1	Adult	Cysts		1:2	1:4*	N.D.	–	–
D.T. 2	2	14	"	11	1:8	N.D.	Neg.	–	–
D.T. 3	3	1	"	5	N.D.	N.D.	+	–	–
	4	1	"	7	N.D.	N.D.	+	–	–
	5	1	"	8	N.D.	N.D.	+	–	–
	6‡	1	Oocysts	6	N.D.	1:6	+	–	–
	7‡	1	"	9	N.D.	1:16	+	–	–
	8‡	1	"	12	N.D.	1:16	+	–	–
D.T. 4	9§	5	"	34	<1:2	1:8000	+	–	–
	10§	5	"	40	<1:2	1:16,000	+	–	–
	11§	5	"	40	<1:2	1:8000	+	–	–

N.D., not done.

* 50 and 60 days after feeding of *Toxoplasma* cysts.

‡ *Toxoplasma* oocysts + *I. felis*.

§ *Toxoplasma* oocysts + *I. felis* + *I. rivolta* (cat).

DISCUSSION

The hypothesis that first linked *Toxoplasma* to the eggs and larvae of the nematode *Toxocara cati* (7–9) was based on only a few observations and thus did not exclude the possible association of a new form of *Toxoplasma*. It was subsequently shown that *Toxoplasma* existed in cat feces independently of *Toxocara* (1, 5). After consistently finding *Isospora bigemina*-like oocysts in the feces of cats fed *Toxoplasma*, we decided to subject this new candidate form to the following mutually independent tests to accumulate critical evidence for or against its identicalness with *Toxoplasma*. (*a*) Use of newborn kittens and littermate controls to avoid as far as possible preexisting coccidial infections. (*b*) Comparison of the development of oocysts and of infectivity in relation to

heat, cold, oxygenation, and chemicals. (*c*) Comparison by filtration of the size of the infectious entity with oocyst size. (*d*) Comparison of the density characteristics of oocysts and of infectivity. (*e*) Comparison of the electrophoretic characteristics of oocysts and infectivity. (*f*) Antigenic comparison of oocysts with the standard RH strain of *Toxoplasma* by means of the fluorescent antibody test. (*g*) Identification of the endogenous cycle preceding the development of oocysts, and linking it antigenically to *Toxoplasma* by specific fluorescent antibody test. (*h*) Comparison of the *Toxoplasma* infectivity of oocysts before and after excystation. (*i*) Comparison of the appearance of oocysts and *Toxoplasma* infectivity in feces of cats after feeding of cysts, trophozoites, and oocysts.

Experimental Findings

The simultaneous excretion of oocysts and of *Toxoplasma* infectivity had been shown in adult cats (Table I). However, the possibility of activating a latent coccidial infection had to be considered. This was minimized by feeding *Toxoplasma* cysts to 1–2 day-old kittens and by observing littermates as controls. Oocysts, *Toxoplasma* infectivity, and coccidian stages were found only in the kittens fed *Toxoplasma* cysts (Table II). Spontaneous coccidial infection in 1–2 day-old kittens is considered unlikely, especially as the mother cats were free of oocysts; indeed the control kittens housed with the infected kittens did not excrete oocysts nor were coccidian stages found in their gut tissues at autopsy. From a procedural point of view we rejected for such critical initial experiments the use of adult cats, even if they were so-called specific pathogen free (SPF). Even in small animals which are more easily controlled, protozoa have been found: *Pneumocystis* and fungi in SPF rats, *Encephalitozoon* in SPF mice, and *Cryptosporodium* in guinea pigs otherwise free of intestinal pathogens, as well as numerous viruses (10–12). The presence of low-grade chronic infections is easily overlooked, and freedom from infection appears most likely at birth, diminishing progressively thereafter.

Numbers of oocysts and infectivity titers proved similar in 122 comparisons (Tables III–VIII). This association was consistently found in the feces of different cats examined on different days after being fed *Toxoplasma* cysts (Table III).

Two types of enumeration problems were encountered. In some instances infectivity was detected before sporulated oocysts were seen (Table VI). The greater sensitivity of mouse inoculation compared with visual examination for the detection of oocysts appears to account for this discrepancy (Table III). Also, infectivity was generally lower than the counted number of oocysts. (Table IV). However, comparisons between fed and excreted oocysts and oral and intraperitoneal titrations indicated that a variable proportion of the inoculum passed through the short gut of mice. Freshly shed unsporulated oocysts were of course not associated with infectivity but as the sporozoites developed within the oocysts, *Toxoplasma* infectivity also appeared (Table VI).

Chemicals that prevented bacterial and fungal growth generally permitted both higher percentages of sporulation and increased *Toxoplasma* infectivity. Low sporulation rates and low infectivity levels of 0.1% formalin, in 20% ethanol, and in water were correlated, as were high sporulation percentages and the development of infectivity in 1 or 2% sulfuric acid or 2.5% potassium dichromate solution. However, neither oocysts nor infectivity developed in 0.3% formalin, 1% ammonium hydroxide, or 1% iodine in 20% ethanol (Table IV), and higher concentrations of these agents might be useful for chemical disinfection (1).

Oocyst sporulation rates in 1 and 2% sulfuric acid or in 2.5% potassium dichromate solution were similar; however, infectivity was usually lower in potassium dichromate-preserved material (Table V). These differences appear to be related to the persistence after repeated washings of dichromate in the oocyst wall with consequent inhibition of enzymatic digestion as has been observed in histochemical procedures (13).

Temperature affected oocyst sporulation and the development of *Toxoplasma* infectivity to the same degree. Both were progressively slowed from 25°–15° and 11°C, and at 4°C no development was observed for 60 days (Table VI). Exposure of feces to 37°–45°C for 60 min did not significantly affect oocyst sporulation or the development of *Toxoplasma* infectivity (Table VIII). But both were killed by 10 min exposure to 50°C and by 24 hr exposure to 37°C (Table VII).

Availability of oxygen affected oocyst sporulation and infectivity similarly. In well aerated samples infectivity developed within a day, but it took 4 days in nonaerated samples (1). Complete anaerobiosis caused a further delay both in the development of infectivity and in oocyst sporulation (Table IX).

Filtrability of fecal *Toxoplasma* infectivity through rated pore sizes of several filter systems coincided closely with both filtrability of oocyst and oocyst size (Table X). Micules, glass beads, and the Nucleopore plastic filters were especially useful in the critical size range needed to compare oocyst and *Toxoplasma* infectivity. Results of filtration were not entirely consistent with the pore ratings for Mitex, Duralon, and sintered glass filters. These filters consisted of an irregular nylon (Mitex) or teflon (Duralon) fiber weave; also their pores became easily clogged with contaminant fecal particles and thus acted as prefilters for oocysts.

The density characteristics of oocysts and *Toxoplasma* infectivity also coincided closely (Fig. 29). In unfloated feces, they were widely distributed in sucrose gradients, but in a clean suspension infectivity and oocysts were concentrated in a narrow band. The separation of oocysts from cat feces for analytical techniques was difficult. In some fecal samples, oocysts could not be separated from fecal particles even by repeated sugar flotations. Filtration of feces through a series of graded wire sieves and glass bead columns, and treatment

with sodium hypochlorite were useful in cleaning oocysts from fecal debris without affecting fecal *Toxoplasma* infectivity (1). A detailed comparison of the methods used will be presented elsewhere.

Finding an increased infectivity titer after the excystation of oocysts that have passed through the preparatory steps of filtration and density gradient is highly significant. It indicates that infectivity resides in a subunit of the oocyst, the sporozoite. Finding only a fourfold rise instead of the expected eightfold rise in infectivity is due to the low excystation percentage of oocysts in vitro. Although 95–98% sporocysts could be freed from the oocysts, only about half of the sporozoites were seen to excyst in vitro.

Continuous particle electrophoresis showed oocysts and *Toxoplasma* infectivity to be similarly distributed (Fig. 30). Preliminary purification of oocysts was necessary to confine them to a few tubes. Discrepancies between individual oocyst counts and infectivity titers were less than one log and are related to the counting of small numbers of oocysts in drops and to the use of only two to four mice per 10-fold dilution.

An antigenic relationship to a standard *Toxoplasma* strain, such as to the RH strain isolated by Sabin (14), should be demonstrable if the oocyst is the morphologic equivalent of *Toxoplasma* infectivity. Oocysts, sporocysts, and sporozoites were stained with anti-*Toxoplasma* mouse sera in the indirect fluorescent antibody test. This staining could be abolished or markedly reduced after the antisera had been absorbed with the standard RH strain of *Toxoplasma*, thus indicating the specificity of the reaction.

The endogenous coccidian cycle preceding development of the oocysts was identified in the cat gut (Figs. 22–28 and Table II). Typical coccidian stages, schizonts, and male and female gametocytes were found in the epithelium of the small intestine of kittens after feeding them *Toxoplasma* cysts, and were absent in control kittens. Schizonts, female gametocytes, and oocysts in gut smears were stained with *Toxoplasma* antibody. Male gametocytes were few and not identified in gut smears. Nonstaining of sporozoites and of the gut stages in paraffin sections is difficult to explain since the oocyst walls and the *Toxoplasma* trophozoites in mesenteric lymph nodes were specifically stained in the same paraffin sections. It is suggested that the antigen present in oocyst and gut stages may be less stable during preparation of paraffin blocks and slides (60°C for 1–2 hr), or that a different antigenic spectrum might be present in these stages.

The simultaneous appearance and disappearance of oocysts and of *Toxoplasma* infectivity in the feces of cats fed cysts, trophozoites, and oocysts, and the similarity in titers, when present, support the hypothesis that *Toxoplasma* infectivity is associated with the oocyst (Tables I and III). The increasing prepatent periods after feeding cysts, trophozoites, and oocysts suggests that these three stages of *Tozoplasma* are linked in a life cycle.

Cats regularly excreted *Toxoplasma* after eating cysts (23 out of 24 cats), but only irregularly after eating trophozoites (4 out of 9 cats), or oocysts (8 out of 17 cats). These differences in transmission could be due to several factors. First, *Toxoplasma* organisms present in the cysts are resistant to digestion by pepsin and trypsin whereas trophozoites are easily destroyed by these enzymes present in the gut animals (15). Therefore, cystic organisms have a better chance of initiating gut infection than trophozoites. However, even the five cats that did not excrete oocysts after eating trophozoites became infected with *Toxoplasma* (1). Second, since the oocyst and sporocyst walls are more slowly digested by trypsin than the cyst walls, more oocysts than cysts may be passed unchanged through the gut of the cat and become lost in the feces. However, six out of nine cats which did not excrete oocysts became infected. Third, *Toxoplasma* may have become so adapted to transmission by cysts via intermediate host that transmission via oocysts and trophozoites has become less efficient.

Thus we have shown that both isosporan oocysts and *Toxoplasma* infectivity have similar characteristics of density, electric charge, antigenicity, and biologic behavior. Both oocysts and *Toxoplasma* infectivity react similarly to chemicals, to different temperatures, to aeration and anaerobiosis. Also, filtrability of infectivity through a series of filters coincides with oocyst size. The increase in infectivity after excystation provides a means of linking the sporozoites within each oocyst to *Toxoplasma* infectivity.

The entire evidence supports the hypothesis that oocysts and *Toxoplasma* infectivity are identical and we may therefore speak of "the *Toxoplasma* oocyst."

Divergent and Congruent Findings

Hutchison (7) initiated the search for *Toxoplasma* in the feces of cats, and suggested that *Toxoplasma* infectivity resided in eggs of the nematode *Toxocara cati* (8). We later separated *Toxoplasma* from *Toxocara* by means of two critical tests and produced *Toxoplasma* infectivity in worm-free cats (1, 5).

Work and Hutchison (16, 17) described a "new cyst" of *Toxoplasma* in cat feces. Their cyst contained "a slightly granular mass" which developed into "two separate organisms." "No definite structures except for some granules could be seen inside them." Correlation between the new cyst and *Toxoplasma* infectivity was based on titration of a single fecal sample in mice, and on the micro-isolation of four new cysts, which were inoculated individually into each of four mice. All of the mice became infected with *Toxoplasma*. Although the size of the new cyst (8–9 × 13–14 μ) and of our oocysts (10 × 12.5 μ) is similar, there is a marked difference between their 3 × 7 μ interior organisms and our 6 × 8.5 μ sporocysts. No photographs were published in the preliminary report (16). In a subsequent paper new cysts are illustrated (17). Although they appear identical with *Toxoplasma* oocysts, and the measurements of interior organisms taken from their photomicrographs are similar to our sporocysts, the latter differ from the 3 × 7 μ interior organisms. It would appear that either the authors were initially looking at different structures from those they illustrated in their detailed paper, or that they did not measure correctly. We consider the new cyst observed by Work and Hutchison to be unrelated to *Toxoplasma* for the following reasons. (*a*) There are differences between the sizes of interior

organisms and sporocysts. (*b*) The relationship of infectivity with a single micro-isolated new cyst is not valid without inoculation of controls with material from the same sample which does not include a new cyst. The idea that four microisolated new cysts infected four out of four mice would be unusual, since in a titration a calculated single oocyst dose produced infection in only one of six mice; in fact, the titration data suggest that at least 1000 oocysts were associated with each new cyst (17). (*c*) The correlation of new cysts with infectivity based on a titration of only a single specimen is not sufficient to rule out a chance association in numbers.

Kühn and Weiland (18) illustrated oocysts from the feces of five cats which were fed *Toxoplasma*-infected mice but they neither claimed nor proved these to be *Toxoplasma* oocysts. Overdulve (19), and Sheffield and Melton (20) also found infectivity of fecal material to correspond with the presence of oocysts except in a few cases where no oocysts were seen. Coincident with the release of sporozoites from the oocysts, *Toxoplasma* infectivity could be shown in tissue cultures (20). The ultrastructure of sporozoites closely resembled that of the trophozoites of *Toxoplasma* (20).

In a letter to the editor and in a paper published 21 days later, Hutchison, Dunachie, Siim, and Work (21, 22) reported on recovering oocysts and endogenous stages from two cats fed infected mice but not from one control cat. They illustrated a schizont and a gametocyte from the epithelium of two infected cats (22). After recognizing the oocysts as being isosporan (23) they equated the new cyst with the oocysts, and assigned the latter to the *Toxoplasma* cycle. This was based on finding the endogenous stages in adult SPF cats, chosen to exclude the possibility of spontaneous parasitic infection. Although as discussed earlier such adult animals do not provide the assurance attributed to them, all recent observations are compatible with the conclusion that oocysts are part of a *Toxoplasma* cycle.

Taxonomy

Toxoplasma was first described in 1908 form studies of a North African rodent (24) and a Brazilian rabbit (25). For 60 years its life cycle was unknown. Studies of its fine structure (26, 27) suggested a structural relationship to such Sporozoa as *Lankesterella* (28), *Plasmodium* (29), and *Eimeria* (30). Since a sexual cycle had not been found, *Toxoplasma* remained either unclassified or was classified in a separate protozoan class Toxoplasmatea (31).

Finding a sexual cycle in the gut of cats helps to classify *Toxoplasma* as a coccidium of cats. Unlike most other known coccidia which are more or less confined to the gastrointestinal tract, *Toxoplasma* has evolved to multiply extensively in other tissues; this is represented by the proliferative and cyst stages which have been well known for years. Also, unlike most other coccidia which infect only one host, *Toxoplasma* has adapted to multiply in many other hosts, where both proliferative and cyst stages are found.

However, no oocysts or fecal *Toxoplasma* infectivity were found in feces of mice, hamsters, rats, guinea pigs, rabbits, raccoons, a skunk, dogs, opossums, Japanese quail, or chickens, although all of these hosts became infected after being fed *Toxoplasma* cysts (2). With cats as primary hosts, these nonfelines may be regarded as intermediate or foreign hosts.

We have therefore classified *Toxoplasma* in the suborder *Eimeriorina* (6) or *Eimeriina* (32) as a member of the family Toxoplasmidae with the characters of the genus (2). If one considered only oocyst structure, *Toxoplasma* might be

placed in the genus *Isospora*. However, the wide tissue parasitism and the presence of foreign intermediary hosts are additional criteria which set *Toxoplasma* apart from *Isospora* and *Eimeria*. *Toxoplasma* should be retained as a separate genus with the following characteristics: schizogony and gametogony in the gut epithelium of cats; oocysts with two sporocysts, each of which have four sporozoites developing outside of the host; trophozoites multiplying by endodyogeny in many types of cells, leading to the production of cysts with many merozoites, mainly in the brain and muscle; being facultatively heteroxenous in many mammals and birds in which only an asexual extraintestinal cycle has been observed (2).

The *Toxoplasma* oocysts described in the present study resemble in structure those of *Isospora bigemina* of the dog and cat (33, 34). Levine and Ivens (33) critically described the structure of the *I. bigemina* oocyst from dog. Shah (34) mentioned that the sporocyst residuum formed a ball in the cat parasite instead of scattered granules as in the dog parasite (33). In the *Toxoplasma* oocyst the sporocyst residuum was variable and both types were even found occasionally in the same oocyst (Figs. 8, 19).

Isospora bigemina was first described as *Coccidium bigeminum* by Stiles (35) who found it in the dog. Railliet and Lucet (36) described three varieties, from dog, polecat, and cat, naming the latter *Coccidium bigeminum* var. *cati*. Lühe (37) later transferred this "species" to the genus *Isospora*. Nevertheless Wenyon (38, 39), who reviewed the earlier literature, believed that *Isospora felis*, *I. rivolta*, and *I. bigemina* were common to dog and cat. However, Nemeséri (40) found that *I. felis* from dogs was not transmissible to cats and he named the dog form *Isospora canis*. Shah (34) also failed to infect dogs with the *I. felis* of cats. We have shown in the Results section that neither *Toxoplasma* oocysts, nor the *I. felis* or *I. rivolta* of cats are transmitted to dogs. Therefore, all the available evidence shows that dog and cat *Isospora* species are different and should be individually designated. We suggest that the term *Isospora bigemina* be restricted to the dog since it was first isolated from dogs. The term *Isospora cati* Railliet and Lucet, 1891, should designate *Isospora bigemina*-like oocysts from cats; Railliet and Lucet (36) had called them *Coccidium bigeminum* var. *cati*.

The life cycle of *I. cati* needs to be studied in cats under controlled experimental conditions since accounts of its schizogony and gametogony are incomplete and confusing. Wenyon (38, 39) found fully developed *I. bigemina* oocyst in the *lamina propria* of some cats and dogs but only in the epithelium of other cats and dogs. *Toxoplasma* schizonts and gametocytes have been found only in the intestinal epithelium, but trophozoites occur in the lamina propria of cats (Dubey and Frenkel, unpublished). Possibly Wenyon was dealing in some instances with a mixed coccidial infection of *I. cati* and *I. rivolta*. Mahrt (41) found schizonts and gametocytes of *I. rivolta* in the lamina propria of dogs experimentally infected with *I. rivolta* of dog origin; the life cycle of *I. rivolta* of cats is unknown.

Oocysts of *I. cati* from cat feces should be tested for toxoplasmic attributes, specifically the capacity to infect other species of animals (mice) and to elicit *Toxoplasma* antibody. Oocysts with toxoplasmic attributes should be designated as *Toxoplasma gondii*; if no toxoplasmic attributes are found they should be designated as *I. cati*.

If all isolates of *I. cati* (as designated above) were found to possess the biological characteristic of *Toxoplasma*, one might be tempted to substitute the earlier specific designation of *cati* (1891) for *gondii* (1908) and create a new com-

bination of *Toxoplasma cati*. However, even finding 1000 *Toxoplasma* isolates would not preclude the 1001st from showing the biologic characteristics of *Isospora*. Since its nonexistence cannot be proven, *I. cati* may have to remain a *nomen dubium*. While we consider it highly important to know whether the majority of *I. cati* in a given locality are biologically *Toxoplasma* or *Isospora*, their occurrence in nature is not mutually exclusive. We therefore recommend retention of the designation *Toxoplasma gondii*.

SUMMARY

Coccidian oocysts resembling those of *Isospora bigemina* were excreted by cats fed *Toxoplasma*. In order to identify these oocysts with *Toxoplasma* infectivity a number of critical comparisons were made. The appearance of oocysts and *Toxoplasma* infectivity was simultaneous in the feces of 23 of 24 adult cats, 3–5 days after feeding of *Toxoplasma* cysts; in the feces of 4 out of 9 cats, 7–10 days after feeding of trophozoites; and in 8 out of 17 cats, 20–24 days after feeding of cat feces containing oocysts. Oocysts and infectivity were present in similar numbers, and they disappeared simultaneously from the feces of cats. Oocysts and infectivity were also observed simultaneously in the feces of 9 kittens, 1–2 days old, fed *Toxoplasma* cysts. Oocysts could not be separated from infectivity by filtration, by continuous particle electrophoresis, or by density gradient centrifugation. Excystation of oocysts was followed by an increase in titer of *Toxoplasma* infectivity.

Unsporulated oocysts in fresh cat feces were noninfectious to mice, but oocyst sporulation was associated quantitatively with the development of infectivity at different temperatures and conditions of oxygenation. Maximum oocyst sporulation at 48 hr correlated with the development of maximum *Toxoplasma* infectivity.

1 and 2% sulfuric acid, and 2.5% potassium dichromate were found to be the best preservatives for sporulation of oocysts and for the development of *Toxoplasma* infectivity. Low sporulation rates in 0.1% formalin, 20% ethanol, and in water were associated with low infectivity in these reagents. Neither *Toxoplasma* infectivity nor oocysts developed in 0.3% formalin, 1% ammonium hydroxide, or 1% iodine in 20% ethanol.

Oocysts, sporocysts, and sporozoites were stained specifically with *Toxoplasma* antibody in the indirect fluorescent antibody test. Typical coccidian stages, schizonts, and male and female gametocytes were found in the epithelium of the small intestine of kittens fed *Toxoplasma* cysts. The classification of *T. gondii* is discussed in relation to that of other isosporan coccidia of cats and dogs. The term "*Toxoplasma* oocyst" is introduced and *Toxoplasma* is classified in the family *Toxoplasmidae* of the suborder *Eimeriina*. The species *Isospora bigemina* is restricted to dogs, and *I. cati* to cats. *I. felis* and so-called *I. rivolta* from cats were noninfectious to dogs, and did not confer immunity to subsequent infection with *I. canis* and *I. rivolta* from dogs.

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