both research and vaccine production. Attenuation of virulence of the virus for chicks by tissue culture may prove to be desirable for immunization purposes.

The diagnosis of an acute AE infection in chicks is thus far mainly based on clinical observations and histopathologic findings. Difficulty in differentiating AE from Newcastle disease and neurolymphomatosis on a histopathologic basis has been encountered. The diagnosis of inapparent infection in breeding flocks by embryo susceptibility test has been employed(8). The test requires 3 weeks to complete and is costly. Collecting representative eggs from large commercial farms and the shipment of them to laboratories may present a problem at times. A one-tube *in vitro* virus neutralization test using 100 $TCID_{50}$ might serve as a simple method for diagnosis of acute AEV infection in chicks and inapparent infection in adults. IC challenge and virus neutralization tests in chicken embryos have been used for evaluation of immunity in inoculated flocks(6,9). The former method is considered too severe and only a fraction of birds can be challenged; the latter method has the disadvantage of requiring a source of susceptible embryos. The virus neutralization test in a cell culture system may serve as a means of evaluating the immunity status of vaccinated flocks without the disadvantages mentioned.

Summary. Virus synthesis, cytopathogenicity, and modification of virulence for chicks, of AEV in chick kidney cell culture after 10 serial passages, have been described. The cell culture virus has been identified by histopathologic changes in inoculated chicks and virus neutralization tests in a cell culture system. The possible application of the findings are discussed.

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Plaque Assay of *Toxoplasma* on Monolayers of Chick Embryo Fibroblasts.*† (25275)

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The importance of *Toxoplasma gondii* as a parasite pathogenic for man and for many animal species is documented in voluminous literature dealing with various aspects of epidemiology, pathology, clinical findings, diagnosis and treatment(1,2). Relatively less is known about the organism as an independent entity. Its classification as a protozoon is based largely on morphological considerations (3). Although experimental infections of various animals and tissue cultures(2) have been studied in considerable detail, we know nothing about the attributes of the parasite responsible for its extremely fastidious requirements for survival and growth. To date, it has not been possible to propagate *Toxo-*

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plasma outside of living susceptible cells, and there is no way of assuring quantitative survival in cell-free media. Therefore, it has been difficult to study its metabolism and mode of multiplication, or, indeed, to establish criteria for viability of the organism. Because, operationally, *Toxoplasma* is an obligatory intracellular parasite, experimental work has largely been patterned after that concerned with viruses. The key to rapid progress in virology has been the gradual evolution of increasingly sensitive and quantitative assay methods. Of special usefulness is the plaque assay described first for bacteriophages(4) and then adapted for use with animal viruses by Dulbecco and Vogt(5). Availability of a similarly quantitative assay for work on *Toxoplasma* would seem to be a prerequisite for systematic investigation of the organism at a fundamental level. The demonstration of cytopathogenic effects produced by *Toxoplasma* in several different kinds of cultured mammalian or avian cells(2) suggested that a plaque system could be developed. Our experiments confirmed this expectation and led to the definition of several quantitative parameters.

Materials and methods. RH strain of Toxoplasma gondii was obtained from Dr. Leon Jacobs of National Institutes of Health. For maintenance, it was subjected to serial intraperitoneal passages in Swiss mice at regular intervals of 4 days. To obtain the organism from infected mice, peritoneal exudate was obtained by injecting 2.5 ml of growth medium (see below) into the peritoneal cavity and aspirating the fluid contents with a syringe. After defibrinating by rolling with glass beads for 5 minutes, the suspension was expelled repeatedly with force through a 27 gauge needle against the inner wall of a test tube to free intracellular parasites. The success of this procedure was verified by microscopic examination which revealed very few intact cells, but millions of extracellular parasites. The same method was used for artificial liberation of intracellular organisms from infected chick embryo fibroblasts. *Chick embryo fibroblast monolayers (CEF)* were prepared according to the method of Dulbecco and Vogt(5). Trypsinized cell suspensions were prepared from 9-day-old chick embryos. Growth medium consisted of Hanks balanced salt solution (Hanks BSS) containing 20% horse serum (Ho) and 5% chick embryo extract (EE). For establishment of monolayers, 5 ml of growth medium containing 3×10^7 cells were placed in a 50 mm Petri dish. After 15 to 24 hours of incubation in a humidified atmosphere of 3% CO_2 in air at 37°C, confluent sheets were ready for infection. *Infection of monolayers.* After removal of growth medium, the monolayers were infected with 0.5 ml of *Toxoplasma* suspension. Different suspending media were used, and varying periods of time were allowed for adsorption of the organisms, as will be described. Adsorption took place at 37°C. At the end of adsorption period, the monolayers were overlaid with 5 ml of agar medium either directly or after removal of the inoculum and washing with phosphate-buffered saline (PBS). The overlay medium consisted of 0.5% bovine albumin, 0.1% yeast extract, 10% Ho, 5% EE in Earle's balanced salt solution (Earle's BSS), 0.9% agar and neutral red at final concentration of 1:30,000.

Results. Plaque production by Toxoplasma. Discrete round plaques, averaging 0.34 mm in diameter, were first visible on fourth day after infection with *Toxoplasma* suspension. Over the following 4 days, they grew to 1.2 mm in diameter. Centrifugal growth became progressively slower, suggesting more rapid destruction of cells infected initially than later. Because of their small size, up to 500 plaques could be counted easily on a single plate 5 days after infection. Plaque counts did not increase significantly after the fifth day. When plaques were "picked" and passed either to mice or to monolayers, infection was always produced. In contrast, no infectious organisms could be recovered from areas of monolayers not showing plaques. On microscopic examination, the plaque presented a patch of degeneration and necrosis containing many thousands of crescentic or oval toxoplasmata. The line of demarcation between neutral red stained, uninfected cells and unstained debris was made up of several rows of cells laden with toxoplasmata.

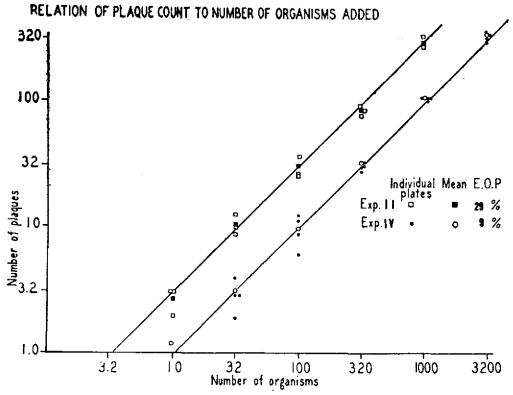

FIG. 1.

Correlation of plaque count with number of organisms plated. Ability to count toxoplasmata directly in a hemocytometer made it easy to calculate the efficiency of plating (E.O.P.) and to demonstrate a linear correlation between number of organisms plated and number of plaques produced. The results of 2 experiments are presented graphically in Fig. 1. That the mean plaque counts fell on straight lines drawn at 45° angles to the abscissa (number of organisms plated) signified that each plaque was induced by infection of a single cell with a single organism(5). In random samples of *Toxoplasma* suspensions, derived from peritoneal exudate of mice (see Materials and methods), the E.O.P. varied between 5.4 and 53% of the counted organisms. An alternate method giving a predictable E.O.P. of about 50% will be described below.

Reproducibility of plaque counts. Degree of reproducibility was tested by replicate plating of 500 parasites on each of 60 monolayers. The suspending medium consisted of 15% EE in Hanks BSS. Thirty plates were overlaid at 90 minutes and the remaining 30 at 180 minutes after infection. Adsorption for 90 or 180 minutes yielded almost identical numbers of plaques. Standard deviations of means for random groups of 5 plates were equivalent to ± 15.8% and ± 14.2%, respectively. Standard deviations from the mean based on counts for 30 *individual* plates were ± 18 or ±18.8%. It was postulated that 95% of replicate samples should give plaque counts reproducible within a range of ± 30 to ± 40% of the mean and that samplings from kinetic experiments were to be considered as comparable if their plaque-forming titers fell within these limits.

Survival of Toxoplasma in various media. Because toxoplasmata die within a few hours in suspending media without added serum or broth(6), a study was made to determine which media were most satisfactory with regard to quantitative survival and E.O.P. Organisms harvested from the peritoneal cavity of an infected mouse were diluted and suspended in various media at a concentration of $2.5 \times 10^3/0.5$ ml or $1 \times 10^3/0.5$ ml. These suspensions were incubated at room temperature. Assays for plaque forming units (pfu) were made initially and at 3 to 4, 6, and 24 hours by plating 0.5 ml and adsorbing to the monolayers for 90 minutes before overlaying.

The results of 2 such experiments are summarized in Table I. Plaque counts obtained with freshly prepared suspensions were comparable within the described limits of reproducibility (see above), and therefore loss of viability in subsequent samples was expressed relative to the initial *mean* plaque counts (Table I). Of the media used, Hanks BSS was the least favorable medium, in that 99% of the pfu were lost after 3 hours. In bovine amniotic fluid (BAF), in Earle's BSS containing 0.5% lactalbumin hydrolysate and 0.1% yeast extract (LaYe), and in 5% Ho in Hanks BSS the number of viable organisms decreased by 50% or more in 3-4 hours. On the other hand, media enriched with EE in concentrations of 5 to 50% preserved 80 to 100% of plaque forming activity for at least 3 hours, and 50 to 60% for 6 hours. However, after 24 hours, plaque forming activity had decreased by over 90% regardless of the nature of the medium. All subsequent adsorption studies were made in Hanks BSS containing EE at concentrations from 5-15%.

Adsorption of plaque forming units (pfu). Preliminary data indicated that the same number of plaques was obtained after 90 or 180 minutes of adsorption (see above). More detailed information concerning time required for adsorption of a maximum number of pfu was obtained by overlaying plates at frequent

TABLE I. *In Vitro* Survival of *Toxoplasma* in Different Media at Room Temperature.

		Time of incubation						
		Initial	3-4 hr		6 hr		24 hr	
Exp. No.	Medium	Po	Pt	Pt/Po	Pt	Pt/Po	Pt	Pt/Po
I	Ho20EE5	351	237	.77	—	—	17	.055
	BAF	335	168	.54	—	—	2	.0065
	LaYe	286	122	.39	—	—	2	.0065
	Ho5	299	87	.28	—	—	0	0
	Hanks BSS	276	3	.0097	—	—	0	0
	Initial mean	309						
II	Ho20EE5	135	95	.66	60	.42	2	.014
	EE5	155	127	.89	68	.48	3	.021
	10	162	126	.88	79	.55	5	.035
	15	155	126	.88	74	.52	4	.028
	20	140	138	.97	77	.54	5	.035
	30	141	132	.92	82	.58	—	—
	50	122	148	1.0	85	.59	5	.035
	Hanks BSS	137	2	.014	0	0	0	0
	Initial mean	143						

For description of media see text. Numbers besides ingredients refer to percentages included in Hanks BSS.

Pt = No. of plaques at time "t." Pt/Po = Fraction of initial mean pfu surviving at time "t." — = not tested.

intervals after infection. The results of 6 such experiments are summarized in Fig. 2. In each experiment, plaque counts obtained on plates adsorbed for 70 to 90 minutes or longer were within the limits of reproducibility previously determined for replicate platings of single samples (± 30% of the mean for multiple plates). The mean of all counts falling within these limits was considered as the maximum (P_m). Fig. 2 presents on a log scale the plaque numbers at times t (P_t) relative to P_m. From the resulting average values, fractions of unadsorbed pfu ($1 - P_t/P_m$) were calculated and plotted (Fig. 3). The curve suggested a slow rate of adsorption for the first

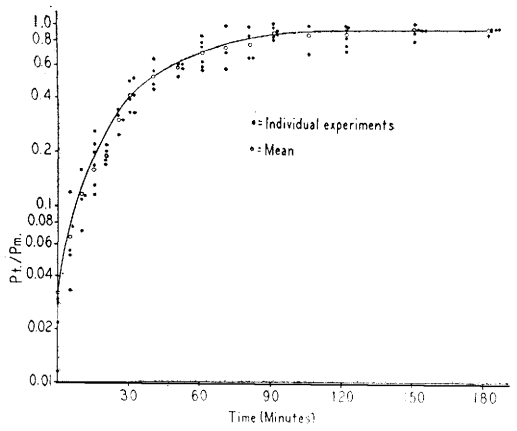

FIG. 2. Increase in No. of plaques with increased period of adsorption.

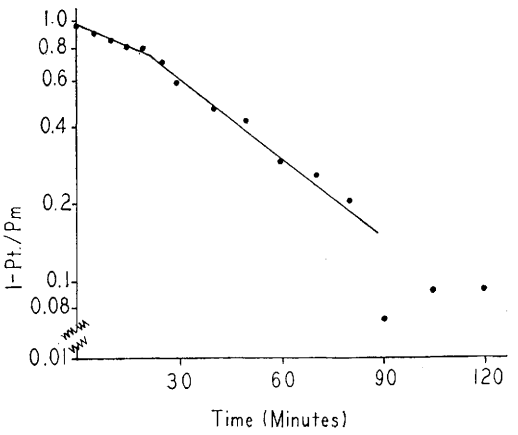

FIG. 3. Decrease of plaque-forming units with increased period of adsorption (calculated from Fig. 2).

20 minutes followed by a more rapid exponential decrease of free pfu between 20 and 80 minutes. The validity of this slope was confirmed by direct assay of unadsorbed organisms in experiment Fig. 4. The velocity constant (Kc) of adsorption between 20 and 80 minutes was calculated from these curves according to the function $P_t/P_m = 1 - e^{-Ket}$ (7) and proved to be Kc = 0.024. This indicated that 9.6×10^{-10} pfu were adsorbed/cell/minute.

Effect of washing, centrifugation, and increased viscosity on adsorption. (a) *Washing.* In several experiments, the effect of

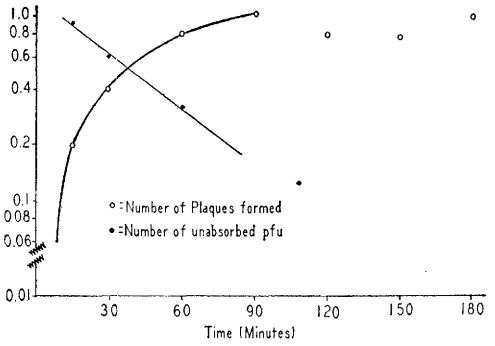

FIG. 4. Experimental determination of slopes for adsorbed and unadsorbed plaque-forming units.

washing monolayers before overlaying was studied. The P_m and rate of adsorption of washed plates were identical in each instance with those on unwashed parallel plates. (b) *Increased viscosity and centrifugation.* Rate of adsorption of toxoplasma was significantly slower than that reported for a number of viruses(7,8). Toxoplasmata are unicellular organisms measuring 6 x 2 μ in size and tend to settle out on standing. In this respect, they differ from viruses, and it seemed possible that prolonged incubation might permit spontaneous sedimentation of organisms out of suspension onto the cell layers.§ This effect should be counteracted by increasing the viscosity of the adsorption medium, intensified by centrifugation during adsorption. Preliminary experiments have confirmed these expectations.

The results shown in Table II were obtained when one group of plates was infected with organisms suspended in the usual medium (15% FE in Hanks BSS). Another group of plates was infected with an aliquot of the same suspension to which 0.4% carboxymethylcellulose (CMC) had been added to increase viscosity of the medium from 1.5 to 13 centipoises. It can be seen that this modification reduced the plaque count at 180 minutes to less than 20% of the Pm which was reached at 90 minutes in the less viscous medium.

As part of the same experiment, some plates, infected in presence or absence of CMC, were centrifuged for 15 minutes at 16 x g and overlaid at the same time as the 15 minute plates which had not been centrifuged. The results (Table II) showed a 7- to 10-fold increase in P_t/P_m in plates subjected to centrifugation.

§ The depth of the liquid layer (0.5 ml in a 50 mm Petri dish) during adsorption is approximately 250μ.

TABLE II. Effect of Viscosity and Centrifugation on Rate of Adsorption of Toxoplasma pfu.

	Without CMC*		With CMC*	
Adsorption time (min.)	No. plaques	Pt/Pm†	No. plaques	Pt/Pm†
15	22	.05	9	.02
30	128	.29	25	.06
60	271	.62	47	.11
90	396	.91	57	.13
120	423	.97	75	.17
180	492	1.10	83	.19
15 centr.‡	154	.35	91	.21

Inoculum 1,000 toxoplasmata/3.0 ml.
* CMC: 0.4% carboxymethylcellulose (see text).
† Pm (mean 90-180 min.) = 437.
‡ Plates centrifuged at 500 rpm (16 × g) for 15 min. during adsorption. Compare with line 1 (15 min. controls).

Although these findings suggested a role of sedimentation in determining the kinetics of adsorption, further work is indicated to separate this effect from that of diffusion, convection(9) and of the spontaneous motility of *Toxoplasma.*

Attempts to standardize E.O.P. As mentioned above, random samples of toxoplasmata harvested from peritoneal exudate of mice had an E.O.P. ranging from 5.4 to 53%. Attempts were made to obtain suspensions yielding consistently high E.O.P. by synchronous lysis of CEF monolayers seeded with enough toxoplasmata in liquid medium to infect each cell. However, less than 1% of the organisms produced in the cells were spontaneously released into the medium at 20 to 100 hours after infection. The cells making up these monolayers were scraped into suspension and lysed artificially as described under Materials and Methods. Under these conditions, fewer than one-third of the liberated organisms were plaque formers.

These findings prompted detailed study of the multiplication of Toxoplasma in CEF monolayers under liquid medium. Cell sheets were exposed to 3 x 10^7, 3 x 10^5, or 3 x 10^3 organisms/ml. The E.O.P. of the infecting suspension was 10%, resulting in effective multiplicities of infection of 1.2 x 10^{-1}, 1.2 x

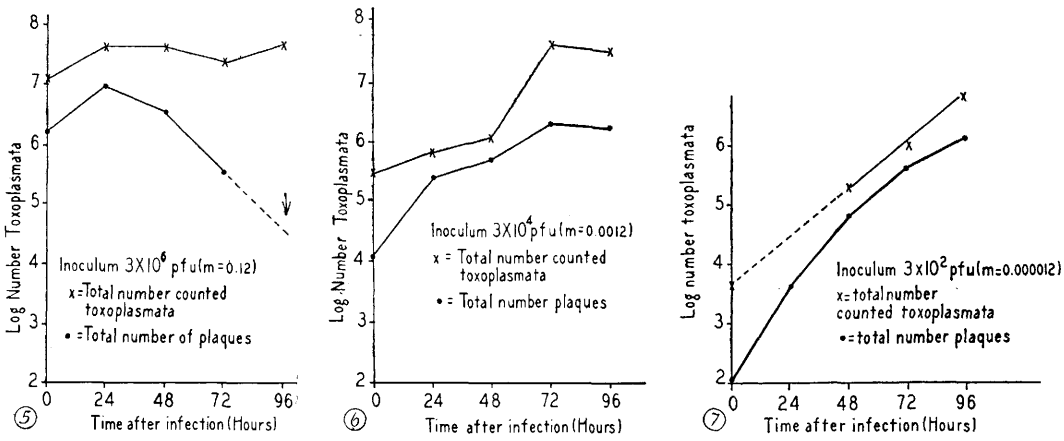

FIG. 5-7. Multiplication of Toxoplasma in chick embryo fibroblast monolayers infected at different multiplicities.

10^{-3}, and 1.2 x 10^{-5} pfu/cell.‖ After 2 hours adsorption, each plate was washed with a total volume of 9 ml of 15% EE in Hanks BSS. The washing fluid was replaced in each plate by 5 ml of medium containing 5% Ho and 15% EE in Hanks BSS. The plates were placed into the CO_2 incubator. At 24 hour intervals, the parasites in the medium and those artificially released from cells were counted and assayed on fresh CEF monolayers.

The results confirmed previous findings that very few toxoplasmata were released spontaneously into the medium. On the other hand, yields of organisms and pfu from artificially lysed cells showed considerable dose dependence. The largest of the 3 inocula (Fig. 5) failed to induce a significant increase in number of counted organisms, although a slightly increased E.O.P. at 24 hours suggested that some multiplication had occurred. At subsequent intervals, the number of organisms remained unchanged, but the E.O.P. decreased progressively, indicating death of the parasites.

In plates infected with 3 x 10^{4} pfu (Fig. 6), the parasites multiplied to a peak number of 5 x 10^{7} (*i.e.*, average of 2/cell) after 72 hours. At this time, the E.O.P. was only 4.4%, although at 24 and 48 hours it had been around 50%.

The most encouraging results were obtained with monolayers infected at a multiplicity of 1.2 x 10^{-5}/cell (Fig. 7). Here, the number of both parasites and pfu increased exponentially and roughly in parallel up to 96 hours after infection when the experiment was terminated. Under these conditions, high yields of organisms with an E.O.P. of 40 to 50% were obtained.

Subsequent experiments with infection at low multiplicity have confirmed the latter observations so that we have been able to produce at will large populations of toxoplasmata of which about one-half are plaque formers.

Discussion. Infectiousness is the only usable criterion for viability of Toxoplasma. Previous methods of infectivity titrations, whether in animals(10,11) or in mass tissue cultures(11), have given results too variable to provide a suitable basis for quantitative studies on the organism itself. Eyles and Coleman(10) found that one LD_{50} was equivalent to 1 or 30 organisms, depending on whether the suspending fluid was serum-saline or saline solution alone. On the other hand, Chernin and Weller(11) compared the LD_{50} in mice with the ID_{50} in mouse embryo roller cultures, using for inoculation toxoplasmata harvested from tissue cultures. Titers obtained by the 2 methods differed by as much as 100-fold. Only 1 out of 56-540 parasites (1.8-0.19%) was detected by the

‖ At time of infection, monolayers contained on the average 2.5 x 10^{7} cells. Of the seed inoculum of 3.0 x 10^{7} cells, 3 x 10^{6} were accounted for in the growth medium.

ID_{50} method, and 1 out of 1-36 organisms (100-2.5%) by the LD_{50} method.

In contrast to these methods, the plaque assay is in effect a direct viable cell count (accuracy obtained with only a few plates is at least equal to that obtained by use of a large number of animals in calculating the LD_{50}). In addition, plaques can be detected in 4-5 days, regardless of size of inoculum, whereas mice inoculated with limiting dilutions die only after about 12 days. It has been demonstrated that each plaque is induced by a single parasite, and that large populations of toxoplasmata can be produced at will in which 1 of every 2 organisms is a plaque former.

One problem which has not yet been overcome is our inability to release more than an average of 2 parasites/cell. In view of the fact that chick embryo fibroblasts which line the plaques usually contain a large number of organisms, the low yield under conditions of saturating infections suggests the operation of some yield-limiting mechanism.

The clear definition of individual organisms as infectious units should facilitate further improvement of media with the ultimate aim of supplying the parasite with its needs for extracellular proliferation. Further work is planned leading to purification and biochemical studies of *Toxoplasma*.

Summary. The RH strain of *Toxoplasma gondii* induces formation of necrotic plaques on monolayers of chick embryo fibroblasts overlaid with nutrient agar. Correlation between number of organisms plated and number of plaques produced indicates that each plaque is induced by a single toxoplasma. Survival of toxoplasmata in various media has been tested, and it has been found that Hanks BSS containing chick embryo extract in concentrations of 5 to 50% preserves 80-100% of plaque forming units (pfu) for 3 to 4 hours, 50-60% for 6 hours. Adsorption of pfu on monolayers is maximal after 70 to 90 minutes (Kc = 0.024), can be retarded by increased viscosity of the medium, speeded by centrifugation. Artificial lysis of chick embryo fibroblasts infected at low multiplicity yields populations with a predictable efficiency of plating of about 50% of the counted organisms.

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Assessment of Antral Protective Mechanisms and Intestinal Susceptibility Gradient to Ulcer Formation. (25276)

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Development of peptic ulcer is dependent upon a shift in equilibrium between ulcerogenic factors (secretions of corrosive acid-peptic gastric juices in varying concentrations and volumes), the protective mechanisms (alkaline buffering secretions, *i.e.* bile, pancreatic juice and succus entericus), and bowel susceptibility to ulceration (quantity and character of mucus secretions, mucosal blood supply and regenerative potential, and intrinsic mucosal cellular resistance.) The antral phase of gastric secretion is largely an auto-regulatory system(1) when the antrum occupies its normal position. Potentiation of