

THE UNIVERSITY OF MANITOBA

TOXOPLASMA GONDII: GROWTH CHARACTERISTICS IN CELL CULTURES,
PRESERVATION IN LIQUID NITROGEN, AND STUDIES OF IN VITRO
NEUTRALIZATION IN VERO CELLS BY PLAQUE ASSAY

BY

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ABSTRACT

TOXOPLASMA GONDII: GROWTH CHARACTERISTICS IN CELL CULTURES, PRESERVATION IN LIQUID NITROGEN, AND STUDIES OF IN VITRO NEUTRALIZATION IN VERO CELLS BY PLAQUE ASSAY

Studies of growth of toxoplasma in L, Vero and secondary CEF cells were carried out to determine the relative sensitivity of the cells to toxoplasma and the pattern of growth of toxoplasma in them. Of the three cell systems Vero cells appeared to be more sensitive to T. gondii. The rate and duration of growth of toxoplasma depended on 1) the cell system used, and 2) the input multiplicity of toxoplasma.

In an attempt to determine a precise, reliable and sensitive cell system for plaque assay of toxoplasma, several cell systems were tested. Vero monolayer was found superior to L, secondary CEF and BHK cells for the purpose of assaying toxoplasma.

The necessity for an improvement in preservation of toxoplasma in liquid nitrogen has been long acknowledged. Among the various freeze mixtures tested, the best results were obtained when 5% DMSO and 10% calf serum was used in the preservation of toxoplasma obtained from mice infected 48 hours earlier and stored at liquid nitrogen temperature, as judged by the survivors which were plaque-formers.

Basic aspects of the in vitro neutralization of toxoplasma by a plaque assay was studied for the first time, using antitoxoplasma serum prepared in rabbits, rats and roosters, in the absence and in the presence of guinea pig complement or accessory factor serum.

Rooster antiserum showed greater neutralizing capacity in the absence of guinea pig complement than in its presence, whereas rabbit and rat antisera showed neutralization in the presence of complement but not in its absence. When heat inactivated complement was used, rabbit antiserum manifested maximum neutralizing capacity.

P. T. Shettigara
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TABLE OF CONTENTS

	PAGE
INTRODUCTION	1
LITERATURE REVIEW	6
I. THE PARASITE	6
II. CULTIVATION OF TOXOPLASMA IN TISSUE CULTURE	8
III. PREPARATION OF IMMUNE SERA	9
IV. PRESERVATION OF TOXOPLASMA	11
V. PLAQUE ASSAY	13
VI. NEUTRALIZATION	14
MATERIALS AND METHODS	19
Preparation of parasite suspension	19
Preparation of antisera	20
a) Rabbits	20
b) Rats	20
c) Roosters	21
Tissue culture	22
a) Secondary chick embryo fibroblast monolayers	22
b) Vero cell monolayers	22
c) L cell monolayers	23
d) Baby hamster kidney cell monolayers	23
Accessory factor serum	23
Complement	24
Preservation of toxoplasma in liquid nitrogen refrigerator	24
Plaque assay	25
Neutralization technique	27

TABLE OF CONTENTS CONTINUED

	PAGE
EXPERIMENTAL PROCEDURES AND RESULTS	30
I. GROWTH CHARACTERISTICS OF TOXOPLASMA IN CELL CULTURES	30
Growth of toxoplasma in L cells	Experiment 1 ... 30
	Experiment 2 ... 30
	Experiment 3 ... 32
Growth of toxoplasma in Vero monolayer	Experiment 4 ... 32
Growth of toxoplasma in secondary chick embryo fibroblasts	Experiment 5 ... 35
II. PRESERVATION OF <u>T. GONDII</u> IN LIQUID NITROGEN	38
Preservation of toxoplasma obtained from mice infected 72 hours earlier, and from tissue culture, in liquid nitrogen and plaque assay	Experiment 6 ... 38
Comparative study of the efficacy of preservation in liquid nitrogen of toxoplasma from mice infected 48 hours earlier in various freeze mixtures	Experiment 7 ... 39
III. PLAQUE ASSAY OF TOXOPLASMA	41
Plaquing efficiency of toxoplasma from mice infected 72 hours earlier, with and without artificial lysis in Vero monolayers	Experiment 8.... 41
Plaquing efficiency of toxoplasma from mice infected 48 hours earlier, with and without artificial lysis in Vero monolayers	Experiment 9 41
Comparative study of plaque assay of toxoplasma in Vero, L, secondary chick embryo fibroblasts and BHK monolayers	Experiment 10 .. 44
Experiments to test criteria in proof of plaques	Experiment 11 .. 46

TABLE OF CONTENTS CONTINUED

	PAGE
Stability of toxoplasma at 37°C by plaque assay	Experiment 12 ... 52
Stability of toxoplasma at 37°C by parasite count	Experiment 13 ... 56
Stability of toxoplasma at 5°C by plaque assay	Experiment 14 ... 56
Adsorption rate of toxoplasma in Vero monolayers	Experiment 15 ... 58
IV. NEUTRALIZATION	58
Neutralization of toxoplasma by rabbit and rat antisera	Experiment 16 ... 58
Neutralization of toxoplasma by rabbit and rat antisera, with and without complement	Experiment 17 ... 62
Neutralization of toxoplasma by rabbit antiserum in the presence of complement, inactivated complement and without complement	Experiment 18 ... 66
Neutralization of toxoplasma by rooster antiserum, with and without complement	Experiment 19 ... 68
Neutralization of toxoplasma by rabbit and rooster antisera with and without accessory factor serum	Experiment 20 ... 68
Neutralization of toxoplasma by rabbit antiserum in excess, in the presence of complement, inactivated complement, without complement, and with complement + buffer, by direct assay without dilution	Experiment 21 ... 71
Neutralization of toxoplasma by rabbit antiserum in excess, in the presence of complement, inactivated complement, and without complement by direct assay without dilution	Experiment 22 ... 74

TABLE OF CONTENTS CONTINUED

	PAGE
Neutralization of toxoplasma by rooster antiserum in excess, with and without complement by direct assay without dilution	Experiment 23 ... 76
DISCUSSION	80
SUMMARY	92
BIBLIOGRAPHY	94
APPENDIX	101

LIST OF TABLES

TABLE	PAGE
I. Final concentration of protective solutes	26
II. Plaque assay for survivors of toxoplasma in various freeze mixture and preserved in liquid nitrogen for 20 days	40
III. Plaquing efficiency of toxoplasma from mice infected 72 hours earlier, with and without artificial lysis in Vero monolayers	42
IV. Plaquing efficiency of toxoplasma from mice infected 48 hours earlier, with and without artificial lysis in Vero monolayers	43
V. Plaquing efficiency of different batches of toxoplasma from mice infected 48 hours earlier, suspended in various collecting fluids without artificial lysis	45
VI. Relationship between concentration of toxoplasma and plaque numbers	51
VII. Neutralization of toxoplasma by rabbit and rat antitoxoplasma sera	63
VIII. Neutralization of toxoplasma by rabbit antiserum, with and without complement	64
IX. Neutralization of toxoplasma by rabbit antiserum, with and without complement	65
X. Neutralization of toxoplasma by rabbit antiserum in the presence of complement, inactivated complement and without complement	67
XI. Neutralization of toxoplasma by rooster antiserum, with and without complement	69
XII. Neutralization of toxoplasma by rooster antiserum, with and without complement	70
XIII. Neutralization of toxoplasma by rabbit antiserum, with and without accessory factor serum	72

LIST OF TABLES CONTINUED

TABLE	PAGE
XIV. Neutralization of toxoplasma by rabbit antiserum in excess, in the presence of complement, inactivated complement, without complement and with complement + buffer by direct assay without dilution 75	75
XV. Neutralization of toxoplasma by rabbit antiserum in excess, in the presence of complement, inactivated complement and without complement by direct assay without dilution 77	77
XVI. Neutralization of toxoplasma by rooster antiserum in excess, with and without complement by direct assay without dilution 79	79

LIST OF FIGURES

FIGURE	PAGE
1. Count of extracellular toxoplasma in L monolayer inoculated with 3×10^8 toxoplasma on day 0, at an IM = 100.0	31
2. Count of extracellular toxoplasma in L monolayer inoculated at an IM = 0.001 on day 0	33
3. Count of extracellular toxoplasma in four pairs of L monolayers, each pair inoculated at an IM = 1, 0.1, 0.01 and 0.001	34
4. Count of extracellular toxoplasma in Vero monolayers inoculated with 1×10^8 toxoplasma at an IM = 7.0	36
5. Count of extracellular toxoplasma in secondary CEF monolayers inoculated with 5×10^7 toxoplasma at an IM = 25.0 on day 0	37
6. Relationship between plaque numbers and concentration of toxoplasma	50
7. Stability of toxoplasma at 37°C in PBS-A with 2% calf serum by plaque assay ("48 hours toxoplasma")	53
8. Stability of toxoplasma at 37°C in PBS-A with 2% calf serum by plaque assay ("36 hours toxoplasma")	54
9. Stability of toxoplasma at 37°C in PBS-A with 2% calf serum by parasite count ("48 hours toxoplasma")	57
10. Stability of toxoplasma at 5°C in PBS-A with 2% calf serum by plaque assay ("48 hours toxoplasma")	59
11. Adsorption rate of toxoplasma at 37°C in Vero monolayers	60

LIST OF PLATES

PLATE

PAGE

1. The plaque characteristic of T. gondii (RH) in Vero monolayer as seen on the 12th day after infection 47

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INTRODUCTION

INTRODUCTION

Toxoplasmosis is a world-wide infection which occurs in many hosts such as humans, animals, birds and possibly reptiles (when their ambient temperature is high) and is caused by Toxoplasma gondii.

T. gondii is an obligate, intracellular parasite which can invade and multiply in nucleated cells of all tissues of all mammals and birds. It is an unclassified protozoan, originally referred to Leishmania and later considered to be a member of class Toxoplasmea within sub-phylum Sporozoa. Recently, one phase of its life cycle has been successfully elucidated in the cat using the strain 7741 (originally isolated from sheep), to show that T. gondii is an intestinal coccidian of cats and is faecally spread. It is believed to have evolved to multiply in brain and muscle and in other species of animals, making it possible for carnivorism to become another means of transmission. With the elucidation of this life cycle, there is reason to believe that this organism would be classified under the order Eucoccidia, sub-order Eimeriina.

Antibody prevalence rates vary as much in animals as in man and evidence seems to be that in animals toxoplasma is often not as well tolerated (Feldman, 1968).

Toxoplasma is responsible for considerable economic losses in sheep in New Zealand and England through abortions, stillbirth and high neonatal death rates. Severe outbreaks, with mortality rates as high as 100 per cent have occurred among ranch mink (Watson et al., 1962).

Ample data indicates that the organism is also widespread among wild animals and birds, independently of whether they are carnivorous or herbivor-

ous. It is evident that T. gondii behaves epidemiologically, parasitologically and clinically in similar fashion throughout its strikingly broad host range (Feldman, 1968).

Human toxoplasmosis is naturally of prime importance as far as clinicians are concerned. Although the rate of inapparent infections among humans varies from place to place, clinical cases are said to be at a minimum, owing in part to our ignorance of the real extent of the infection.

Congenital toxoplasmosis is clinically the most important form. About 1-2 births in 10,000 live births are known to be suffering from congenital forms of clinical toxoplasmosis. In these cases a tetrad of signs characterized by chorioretinitis, hydrocephaly or microcephaly, psychomotor retardation, and cerebral calcifications is present. Abortion and stillbirth, either with or without congenital malformation, are also known to be caused by this infection. The pathogenesis of the congenital form is clearer than that of the acquired form. The foetus is infected from the mother via the free stage of toxoplasma which can cross the placental barrier. Maternal infections acquired during the second trimester appear to be of greatest hazard to the foetus. Most workers in this field are agreed that a mother who has given birth to one child with congenital toxoplasmosis will have subsequent pregnancies free of risk. Some do believe that recurrent toxoplasmosis is a cause of foetal loss through habitual abortion and congenital malformations.

Acute acquired toxoplasmosis has gradually unfolded as a clinical syndrome. Initially it was reported to produce both fatal and self-limited encephalitis in young children and a spotted-fever-like syndrome with pneumonia. An apparently benign syndrome marked by lymphadenopathy and relative lympho-

cytosis was subsequently described. In some cases there may be signs of myocarditis and also a diffuse maculopapular rash. Despite this, most infections are asymptomatic and not recognized. It must be remembered, however, that even in the asymptomatic cases organisms persist in cysts in all tissues, probably for the life of the host. This is of importance in the case of people suffering from lymph-node disease and in the case of patients undergoing immunosuppressive therapy - several instances of toxoplasmosis occurring during treatment for leukemia etc., have been reported.

The role of toxoplasma in human uveitis remains a recalcitrant problem. Chorioretinitis is common in the congenital form generally involving both eyes and toxoplasma have been isolated from eyes removed surgically. The problem is whether a mild congenital form of chorioretinitis may be reactivated, or whether an acquired form may be present and whether it reactivates periodically. Specific diagnosis remains difficult.

Congenital (transplacental) transmission in humans is conclusively established. Similarly, pig-to-pig transmission by oral route has been demonstrated (Verma and Dienst, 1965). Carnivorism and faecal spread seem to play important roles in the transmission of toxoplasma among humans. Evidence regarding transmission by arthropod vectors is, rather, inconclusive.

So far, it has not been possible to propagate T. gondii outside of living cells. In their metabolic studies on toxoplasma, Fulton and Spooner (1960) found that toxoplasma was able to oxidize glucose and produce CO₂ by a pathway similar to the Embden-Meyrhof-Parnas scheme of phosphorylating glycolysis. Hexokinase was detectable and toxoplasma was found to contain a cytochrome system. Employing enzyme histochemical study, Capella and

Kaufman (1964) demonstrated the presence of enzymes of glycolysis, namely, lactate dehydrogenase, glucose-6-phosphate dehydrogenase and glycerophosphate dehydrogenase, of Kreb's cycle, comprised of malic dehydrogenase and succinic dehydrogenase and of electron transport chain, that is, reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorases in the mitochondria of both the cyst and proliferative forms. The presence of these enzymes suggests that the metabolic requirements which make toxoplasma unable to survive extracellularly are probably not directly associated with energy production. It has also been recently reported that free toxoplasma are capable of incorporating ³H-uridine for a short time into the three components of toxoplasma ribonucleic acid (RNA), indicating that free toxoplasma are capable of synthesizing RNA (Remington et al., 1970).

While numerous strains of toxoplasma have been isolated both from humans and animals (RH, Beverley, MF, HI, JQ, ESK, Alt, HD, SP3, K65, DX, Weiss, Huff, etc.) all strains recovered in nature, to date, by the methods used to examine them appear to belong to a single species of T. gondii. In vitro cross-neutralization tests using human and hare strains as antigen (Christiansen and Siim, 1951), in vivo neutralization tests by challenging passively immunized mice with toxoplasma, in vitro neutralization of toxoplasma followed by inoculation of mice for death rate and survival times (Nikkels, 1965) and polyacrilamide-gel electrophoresis (Bloomfield and Remington, 1970) employed to distinguish different strains into immunologic types have failed to distinguish the different strains.

The present study was undertaken to develop a precise, reliable and sensitive plaque assay system that can be used to measure accurately the

surviving toxoplasma following neutralization by antisera. The necessity for an improvement in preservation of toxoplasma in liquid nitrogen has long been acknowledged. Preserving toxoplasma in various freeze mixtures at liquid nitrogen temperature, the most promising results were obtained when toxoplasma was preserved in 5% DMSO and 10% calf serum. Comparative study of growth of toxoplasma in different cell systems were carried out to examine the relative sensitivity of the cells to toxoplasma and the pattern of replication therein. Basic aspects of neutralization of T. gondii (RH) by rabbit, rat and rooster antitoxoplasma sera was studied as a prelude to kinetics of neutralization and to cross-neutralization tests by representative antisera against different strains of T. gondii in cell culture that may be envisaged to be employed in future study in order to determine if there is one or several strains of toxoplasma immunologically, since recognition of specificity of the strain of toxoplasma would be of immense help in epidemiological investigations directed not only to tracing back and forth of the source and spread of infection in the event of an outbreak of toxoplasmosis and in transmission studies, but also to relating its specific strain to certain specific clinical entity in etiological studies.

LITERATURE REVIEW

LITERATURE REVIEW

I. THE PARASITE.

T. gondii occurs in two forms: 1) Trophozoite or Proliferative form, measuring 2-4 x 4-7 μ m in size and crescent in shape. Fixation in methanol and staining with Giemsa or Wright's stain render the nucleus red or purple and the cytoplasm pale blue. The anterior end is pointed or motile and the posterior rounded end contains the nucleus. It multiplies usually, if not always, by endodyogeny, that is, division by simple longitudinal fission involving development of two daughter cells within the cell-wall of the mother (Goldman et al., 1958). 2) Cyst form, 30-100 μ m in diameter. Cyst contains numerous individual toxoplasma and is bounded by a double-layered cyst wall. Cysts arise from proliferative forms in later stages of acute infection, during sub-acute and possibly during chronic infection. In the brain and eye, cysts are spherical and in the cardiac and skeletal muscles they are elongate.

The main features, namely, the conoid, paired organelles, convoluted tubes (toxonomes), nucleus with well-developed nucleoli and micropyle, are present in both forms.

Toxoplasma, Coccidia and Plasmodium sporozoites are ultrastructurally similar (Garnham, 1962). Morphologically proliferative forms of Besnoitia are indistinguishable from toxoplasma (Frenkel, 1950).

The final stage of leucocytic parasitization by toxoplasma (as in the leucocytes of peritoneal exudate of infected mice) has been designated as

"terminal colony" (Frenkel, 1956), to distinguish it from the cyst and the proliferative form.

It is accepted that only one species of T. gondii exists and that it is an unclassified protozoan, originally referred to Leishmania and later considered to be a member of class Toxoplasmea within sub-phylum Sporozoa (Westphal, 1954; Feldman, 1968). Recently, with the elucidation of a phase of its life-cycle in the cat using strain 7741, originally isolated from sheep, by three groups of researchers, namely, Frenkel et al., (1970), Hutchison et al., (1970), and Sheffield et al., (1970) independently and almost simultaneously to show that T. gondii is an intestinal coccidian of cats, spread through ingestion of oocysts excreted in cat's faeces, there now seem to be good reasons to classify this organism under the order Eucoccidia, sub-order Eimeriina.

Numerous strains of T. gondii have been isolated from humans, animals and birds since it was first described and named by Nicolle and Manceaux who found it in a North African rodent (Ctenodactylus gondii) in 1908. One of the noteworthy features has been the variation in virulence of some strains of toxoplasma, high virulence of some, and the low virulence of many strains isolated from healthy animals. Erichsen and Harboe (1953) and Harboe and Erichsen (1954) recovered strains of toxoplasma from chickens during an epidemic in Norway and found the organisms isolated, capable of producing the disease on inoculation into the progeny of the same flock. Jacobs et al. (1954) reported isolation of a strain of low virulence on continuous passage in eggs whereas it

became quite virulent when passaged in mice. Lainson (1955) isolated six strains from mice. They were all of low virulence for mice. Two of the six strains were passaged repeatedly in mice for more than a year without any appreciable enhancement of their virulence. However, chance passage in multimammate rats and in canaries caused death of the hosts. Repassage of the strains from the rats and canaries back to mice resulted in fatal infections.

II. CULTIVATION OF TOXOPLASMA IN TISSUE CULTURE

T. gondii was first cultivated in Maximov slides of chick embryonic tissue by Guimaraes and Meyer in 1942 in Brazil. Meyer and de Oliveira (1945) and Muhlfordt (1952) reported cultivation of toxoplasma in avian tissue in vitro. Jacobs et al. (1952) were able to grow the organism in Maximov cultures of chick embryonic heart muscle, leg muscle and in liver epithelium.

Cultivation of toxoplasma in mammalian tissues for the first time was reported by Lock (1953) using embryonic rat heart muscle-explants on coverslips in Carrel flasks under a fluid medium containing 10% each of rat serum and embryonic extract in Tyrode's solution. Chernin and Weller (1954) propagated the parasite in roller tube cultures of mouse embryonic tissues, and human epithelium, myometrium and embryonic skin-muscle tissues. Vischer and Suter (1954) used cultures of macrophages from mice and other laboratory rodents in which they were able to grow toxoplasma to study the role of macrophages from immunized and non-immune animals, and humoral factors in immunity to toxoplasma infection. Various embryonic tissues of chicken, mice and rats, as well as various tissues of

adult rabbits, monkeys and humans were successfully employed for cultivation of T. gondii (RH) by Cook and Jacobs in 1958. Hogan et al. (1961) were able to grow five different strains of toxoplasma in six cell lines, namely, HeLa, retinoblastoma, human nasal epithelium, adult rabbit kidney, embryonic rabbit kidney and rat embryonic fibroblasts. HeLa cells, and HeLa and L cells in Earle's solution were used for morphological studies on the development of toxoplasma cysts by Matsubayashi and Akao (1963). HeLa cell cultures were also used by Lund and Lycke (1963) and Lycke and Lund (1964 a & b) in their study of titration of infectivity and determination of growth rate of T. gondii.

A variety of mammalian and avian cells have been, since, used to cultivate toxoplasma in vitro.

III. PREPARATION OF IMMUNE SERA

Vischer and Suter (1954) immunized rats and guinea pigs by subcutaneous injections of living toxoplasma obtained from the peritoneal fluid of infected mice. One to three injections of 0.5 ml each were given at intervals of three weeks. For the first injection, the peritoneal exudate was diluted 100-fold, for the second, 10-fold and for the third, undiluted. Van Nunen and Van Der Veen (1965) injected three rabbits subcutaneously with about 150 parasites and four rabbits with 90 parasites (apparently with live toxoplasma). From the fifth day of injection they treated the animals with sulphamethazine, 120 mg each intravenously, twice a day for ten days. However six of the seven rabbits were reported to have died in two to four weeks after the onset of infection. According to them the titre reached as high as 1:16,000 both in

the dye test and in the indirect fluorescent antibody test, 28 days after infection.

De Lalla and co-workers (1966) used live, virulent toxoplasma about one million per injection, three injections at intervals of four weeks to immunize rats. For the first injection, 1 ml of Freund's adjuvant was added to toxoplasma suspension and was given subcutaneously. The other two injections were given by intraperitoneal route. Blood was collected 10 to 15 days after the last injection. Some of the rats had received, in addition, 1 ml of exudate previously subjected to osmotic lysis in distilled water for 24 hours.

Rabbits were immunized with two injections given subcutaneously at an interval of 30 days, each injection comprising 1 ml of toxoplasma infected mouse peritoneal exudate which had been kept at 4°C for 15 days in .2% formalin. For the first injection 1 ml of Freund's adjuvant was incorporated with the exudate.

Rabbits were used for obtaining antitoxoplasma serum by Strannegard (1967). He injected rabbits with 1,000 live toxoplasma into the ear vein and bled them 10 days later and obtained a dye-test titre of 1:2,000. In a second paper (Strannegard, 1967b), he reported to have obtained immune serum by bleeding rabbits eight days after an intravenous inoculation of about 50,000 toxoplasma parasites (apparently living toxoplasma). Smuggs et al. (1968) used toxoplasma grown in tissue culture, Ru-1 cells to immunize rabbits. The organisms were suspended in phosphate buffer saline (PBS, pH 7.2) and inactivated with beta propiolactone (BPL) in a final concentration of 0.1%, washed with PBS and resuspended in PBS. Number of organisms per inoculum varied from 1×10^6 to 2×10^8 . For the initial

inoculum 0.25 ml of a mixture consisting of equal parts of inactivated organisms and Freund's adjuvant was given into each foot-pad of rabbits, followed by three intravenous injections of 0.5 ml of inactivated organisms at weekly intervals. One week after the fourth injection, rabbits were bled by cardiac puncture.

Phenol killed toxoplasma was used for immune sera preparation in guinea pigs by Foster et al., (1968) who reported that guinea pigs developed dye test antibodies but that five out of seven died. When adjuvant was used, the antibody titres were higher and six or seven guinea pigs resisted challenge. Guinea pigs were inoculated with 2.5×10^7 organisms in 0.2% phenol per injection, for a total of three inoculations given by subcutaneous route every other day. Freund's complete adjuvant was added in equal parts for the first injection.

IV. PRESERVATION OF TOXOPLASMA

While laboratory strains of toxoplasma are easily passaged by any route of inoculation in mice, rats, hamsters, rabbits, guinea pigs, chick embryos and pigeons, the mouse is by far the most convenient laboratory animal as it is very unlikely to have spontaneous toxoplasma infection. The rat suffers little disease even when inoculated with large numbers of parasites.

Ruchman and Fowler (1951) were able to recover toxoplasma consistently from the brain of rats for as long as two years after inoculation. Jacobs (1956) reported obtaining similar results for up to 24 months. Rat brain can serve as a "bank" for storing toxoplasma. Other "banks" are hibernating animals and cell cultures. Rodhain (1951) infected a hibernating marmot intraperitoneally with a squirrel strain of T. gondii

and the animal died of infection 18 days after the end of hibernation which lasted three months.

Meyer and de Oliveira (1943), 1945) preserved Maximov slide cultures of toxoplasma infected chick embryonic tissue at room temperature for one year and eight months without loss of virulence.

Chandler and Weinman (1956) preserved T. gondii in 15% glycerol-saline at -70°C and observed well over 99% loss of organisms. However, starting with a sufficient number of organisms (1.7×10^7), recovery of organisms in mice was accomplished after storage for 122 days.

Eyles and co-workers (1956) added equal amounts of 10% glycerol-saline to peritoneal exudate from infected mice diluted 1:5 with normal saline containing 20% serum and distributed the mixture in 2 ml amounts in thin glass ampoules which were slowly frozen in a mechanical freezer for 48 hours, after which they were transferred to dry ice-alcohol freezing mixture in a Dewar flask. They found that the organisms survived at least 209 days and they obtained best results using 5 to 10% glycerol.

According to Stewart and Feldman (1965), toxoplasma survived best in the medium used for their culture containing amongst others, 2.5% inactivated calf serum and 10% lactalbumin hydrolysate. In this toxoplasma was reported to have remained viable for at least 90 days at 4°C and as long as 360 days in ice.

Kwantes et al., (1967) added 10% dimethyl sulfoxide (DMSO) and 15% human serum in Hanks' solution to a centrifuged suspension of infected mouse brain and distributed the mixture in 2 ml ampoules which were kept at -70°C . Toxoplasma was reported to have survived thus for two years without change in virulence.

Paine and Meyer (1969) obtained toxoplasma from the pellet resulting from centrifugation of infected bovine kidney cell (MDBK) suspension and culture fluid and resuspended it in 4 ml freeze medium comprising 68% Eagle's minimum essential medium (MEM) with non-essential amino-acids, 25% calf serum and 7% DMSO. The suspension was distributed in one ml amounts into sterile ampoules which were heat-sealed and stored in a liquid nitrogen refrigerator after pre-cooling in a refrigerator to 4°C. Presence of viable, infectious toxoplasma was demonstrated by mouse inoculation and was confirmed by microscopic examination of fresh fluid as well as stained smears of the fluid after 6, 87 and 333 days of storage.

V. PLAQUE ASSAY

Chaparas and Schlesinger (1959) found that the plaque counting method described first for bacteriophages by d'Herelle (1917) and then adapted by Dulbecco and Vogt (1954) for isolation of pure lines of poliomyelitis viruses (PV), could be used for quantitative determination of the infectivity of T. gondii and that the method was more sensitive and yielded a higher degree of accuracy than other methods such as particle counting or quantal response methods such as LD₅₀ and ID₅₀. They employed primary chick embryo fibroblast cultures in which plaques were found visible in five days after infection with toxoplasma.

In their study on the assay of infectivity of T. gondii in tissue cultures and in embryonated eggs Lund et al., (1963) observed that clearly visible plaques caused by toxoplasma appeared in HeLa cell monolayers under the liquid medium on the fourth day after inoculation

when the inoculation dose was large but that the plaques were confluent and hence were difficult to count with accuracy. With smaller sized inocula, they found that the plaques could be counted after 12 days of incubation even without staining with neutral red and that the plaques were one to two mm in diameter. They have also reported development of clearly visible plaques on the chorioallanotic membrane of 12 day-old embryonated eggs after three days of inoculation with 0.1 ml of various dilutions of T. gondii suspension.

Foley and Remington (1969) reported obtaining higher plaquing efficiency and reproducibility accompanying the use of secondary, rather than primary chick embryo fibroblast cultures and toxoplasma obtained from disrupted peritoneal cells of mice infected 48 hours earlier. Plaques were formed in five days as white, irregular areas against a pink background of viable cells.

VI. NEUTRALIZATION

The Dye Test (DT) introduced by Sabin and Feldman (1948) is a quantitative procedure, in which live toxoplasma are suspended in fresh, non-antibody containing normal human serum. Equal amounts of this are then mixed with varying fourfold dilutions of the serum (inactivated at 56°C for 30 minutes), the antibody content of which is being measured. Following incubation for one hour at 37°C, a small amount of alkaline methylene blue is added and the proportion of stained and unstained parasites are estimated in each dilution by examining a wet film. The titre of the serum is the dilution in which 50 per cent of the parasites are unstained.

However, Lelong and Desmonts (1952), in elucidating the nature of the dye test, reported that both in hypotonic saline solutions and after freezing, the toxoplasma do not stain with alkaline methylene blue

and that their behaviour in this and in other respects being similar to that of lysed bacteria which lose their affinity for dyes and become less refractile, they concluded that DT reaction is a manifestation of incomplete toxoplasmolysis.

Christiansen and Siim (1951) performed, among other tests, cross-neutralization tests using as antigen in the dye test, human and hare strains of toxoplasma and found that toxoplasma of hares appeared to be serologically identical with the human R.H. strain besides being equally pathogenic.

In order to determine if various strains of T. gondii differed serologically, Wildfuhr and Hudeman (1952) infected rats with ten different strains of toxoplasma and used their sera as well as sera of some of the human patients, in the dye test againsts parasites of all strains. They found that all the strains were identical serologically but that they differed from each other quantitatively in the titre at which the reaction took place.

Lycke and co-workers (1965) studied the effect of immune serum and activator or accessory factor (AF) on the infectivity of toxoplasma for cell cultures or ability of toxoplasma to penetrate cells. They used standardized cultures of HeLa cells in Gey chambers. The effect of immune serum with and without AF was judged by the relative number of infectious units (RNIU), that is, the ratio between the number of parasites that penetrated the host cells and the number of exposed host cells, using phase-contrast microscopy. They found that the penetration of toxoplasma was inhibited not only when the organisms were exposed to immune serum with AF but also if they had been incubated with immune serum in which

the heat-labile components were destroyed.

Nikkels (1965) in his thesis described the inability to produce either an in vivo neutralization test or an in vitro neutralization test. In the in vivo neutralization or mouse protection test, with ten or 100 toxoplasma, it was found impossible to reduce death rate of mice by passive immunization with rabbit antitoxoplasma serum. In the in vitro neutralization test, each antiserum dilution was mixed with a toxoplasma suspension, incubated at 37°C for one hour and then inoculated into mice. Ten mice were used for each test and their death rates and survival times were measured. The presence of AF was found essential for demonstration of a neutralizing effect, which, however, was variable and often incomplete in that it did not protect all the mice from dying. He also compared the neutralization test with DT and obtained parallel results. He thus concluded that they were detecting the same antibody.

In an interesting study Strannegard (1967b) reported his findings on the immunoinactivation of T. gondii under the electron microscope using ferretin labelled rabbit antibody. He observed that in the presence of AF serum, the parasite membrane was extensively damaged and the antibodies were seen in the interior of parasites, whereas in the absence of AF serum, antibodies were distributed on the surface of the parasites. In a further study, Strannegard (1967a) described the immunoinactivation of T. gondii by specific rabbit and human antibodies at various temperature, pH and ionic strength by noting the morphological alterations of the parasites under phase contrast microscopy. In an earlier study, Strannegard and Lycke (1966) reported the role of properdin in the antibody effect on toxoplasma by the morphological alteration under phase contrast microscopy.

A possible method that may be used for a neutralization study of toxoplasma is the neutralization kinetics method used for determining very close relationship of viruses such as poliovirus, herpes simplex virus and Western equine encephalitis (WEE) virus.

In a series of brilliant experiments Dulbecco et al. (1956) set the foundation for further kinetics of neutralization studies by enumeration assay in respect of WEE and poliomyelitis type 1 viruses. They performed, besides the kinetic and multiplicity curves of neutralization, the effect of temperature, pH and ionic strength on neutralization reaction. McBride (1959) demonstrated, using plaque assay technique, the serologic individuality of each of a large number of poliomyelitis virus strains of the same type. He stated that the serologic specificity of a strain was independent of the culture system in which the strain was propagated, that is, the neutralization rate constant (K) values for virus grown in MK, HeLa and skin cultures were one and the same. He noted that late antisera were somewhat less specific than early antisera as the heterologous normalized neutralization rate constant (NK) values for late antisera were higher than for early antisera. Reporting on the reaction between poliomyelitis virus and neutralizing antibody in rabbit antiserum, Mandel (1961) outlined his impressive observations that neutralization at neutral pH was irreversible, that at pH 2.0 to 2.5, however, dissociation did take place and that neutralization of virus by antibody was not necessarily accompanied by permanent or irreversible changes in the viral particle. Diwan et al. (1963) described a disc neutralization test employed by them to study the antigenic analysis of poliomyelitis viruses, the test being

designed to measure the degree of neutralization of the virus without being influenced by the rate of viral growth. Ozaki et al. (1963) reported the results of their kinetics of neutralization that enabled them to distinguish oral polio vaccine strains from the wild strains. Ashe and Scherp (1963) employed the same method to divide fifteen strains of Herpes simplex virus into four distinct serotypes based on K values which were converted into NK values in the reciprocal neutralization test by plaque technique.

It is interesting to find that under certain conditions, some anti-viral antibodies have little or no ability to neutralize viruses in the absence of complement (Yoshino and Taniguchi, 1965a and b; Taniguchi and Yoshino, 1965).

Studies with herpes simplex virus indicate that the antibody which requires complement for neutralization appears relatively early after immunization and is mainly of the Ig M type (Hampar et al. 1968). It has been shown that herpes simplex virus sensitized with this kind of antibody is neutralized by the addition of relatively large amounts of purified C'4 in the presence of purified C'1 and that in the presence of a limited amount of C'4, increased neutralization can be achieved by the addition of C'2 and C'3 (Daniels et al. 1969). These findings suggest that viral neutralizing antibody, especially in sera from bleedings obtained soon after infection, may be missed unless the neutralization test mixture contains complement.

MATERIALS AND METHODS

MATERIALS AND METHODS

PREPARATION OF PARASITE SUSPENSION

T. gondii (RH)¹ originally isolated from a child suffering from encephalitis by Sabin (1941) was used throughout this study. It was maintained in Swiss Webster mice of four to eight weeks' age, weighing 20 to 26 gms. Infected mice when moribund, were killed and the peritoneal exudate inundant with toxoplasma was collected with a sterile 23 gauge needle and syringe after incising the skin of the abdomen and subcutaneous tissue and exposing the peritoneum aseptically. The exudate was diluted 1-10 with normal saline solution and 0.1 ml of this suspension containing about one million toxoplasma was injected intraperitoneally into each of four mice. Passage from mouse to mouse was carried out every three days for the purpose of maintenance of the parasite for use in the experiments.

Unless otherwise stated, toxoplasma for plaque assay and neutralization experiments was collected from mice which were infected with two to three times the above dose of toxoplasma intraperitoneally 48 hours earlier, in collecting fluids such as normal saline solution, Dulbecco's phosphate buffer solution-A (PBS-A), PBS-A with 2% calf serum, Hanks' balanced salt solution (HBSS), HBSS-A, and Growth medium (RIF)² specified under each experiment, plus 200 units/ml penicillin, 200 µg/ml streptomycin and 2.5 µg/ml amphotericin B (Fungizone) (PSF) in a sterile stoppered

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1. T. gondii was kindly supplied by Dr, Kenneth Walls, Communicable Disease Center, Atlanta, Georgia.
 2. Dr. A. M. Wallbank, personal communication.

glass tube with sterile glass beads. The glass tube was kept in ice bath at all times. After rolling with glass beads for about five minutes to defibrinate, the count of live, extracellular toxoplasma was made in a Neubauer-Levy haemocytometer after a 1-10 dilution of toxoplasma suspension in alkaline methylene blue dye solution in a separate test tube.

PREPARATION OF ANTISERA

a) Rabbits:- Two rabbits were immunized by two subcutaneous injections of formolized toxoplasma. Toxoplasma obtained from the peritoneal exudate of mice infected 72 hours earlier was collected in PBS-A, and to nine parts of this suspension one part of 1:400 formalin (diluted in PBS-A) was added to make a final concentration of 1:4,000 formalin. It was allowed to stand at room temperature (21 to 23°C) for one hour and was kept at 4°C for a fortnight.

First injection consisted of 0.5 ml each of Freund's complete adjuvant and formolized toxoplasma suspension containing 6×10^6 toxoplasma, given subcutaneously at five different points at the upper nuchal region. Second injection of 0.5 ml formolized toxoplasma suspension was given after an interval of 30 days and the rabbit was bled by cardiac puncture under ether anaesthesia, eight days after the second injection.

b) Rats:- Two rats each were immunized intraperitoneally and subcutaneously with 0.2 ml of toxoplasma suspension in PBS-A containing about 1×10^6 live toxoplasma obtained from mouse peritoneal exudate. First three injections were given on alternate days and the booster injection on day 21. Rats under ether anaesthesia were bled on the 30th day by cardiac puncture.

c) Rooster:- Two roosters were used for immune sera preparation. One rooster was given seven intramuscular injections of live toxoplasma obtained from mouse exudate and collected in PBS-A, the dose of each injection being 0.5 ml containing about 1×10^6 toxoplasma, given intramuscularly into the breast muscle. The first six injections were given on alternate days, the final injection on day 21 and the rooster was bled by cardiac puncture 14 days after the final injection.

The second rooster was immunized intravenously with live toxoplasma obtained from mouse exudate and suspended in PBS-A, and 0.5 ml amounts of toxoplasma suspension was injected each time on days 1, 6, 11, 18 and 31, respectively, containing about 1×10^6 , 2×10^6 , 3×10^6 , 4×10^6 and 5×10^6 toxoplasma in 0.5 ml. The rooster was bled by cardiac puncture ten days after the final injection.

One or two days before commencement of the immunization schedule, blood was collected for preinoculation sera from the ear vein in rabbits, by cardiac puncture under ether anaesthesia in rats and from the wing vein in roosters.

Blood was always collected in sterile centrifuge tubes, allowed to clot at room temperature for about an hour, and was centrifuged at $2,400 \times g$ for 30 minutes. Serum was decanted into another centrifuge tube which was spun at $1,000 \times g$ for 20 minutes. All serum samples were sterility tested in AC medium³ and Sabouraud's broth³. In the event of any contamination, the serum was filtered through Millipore membrane of $0.3 \mu m$ pore-size, tested for sterility as before and kept at $-20^\circ C$.

All preinoculation and immune sera were inactivated at 56°C for 30 minutes just prior to use in neutralization experiments. All batches of calf serum used in the growth medium and in the agar overlay and all preinoculation sera were tested for the presence of toxoplasma antibodies by indirect fluorescent antibody test (IFAT) and were found negative.

The IFAT titres of the antisera used in neutralization experiments are given under each experiment.

TISSUE CULTURE

a) Secondary chick embryo fibroblast monolayers: Primary cultures of chick embryo fibroblast (CEF) were prepared by the method used by A. M. Wallbank⁴. Trypsinized cell suspensions were prepared from nine to ten day-old chick embryos. Monolayers formed by primary cells were trypsinized using a mixture containing 0.02% trypsin solution in PBS (pH 7.3) and 1:5,000 ethylenediaminetetraacetic acid (EDTA) for about ten minutes at room temperature, centrifuged for ten minutes at 250 X g and the cells were resuspended in growth medium to contain 5×10^5 cells/ml for seeding 60 mm petri dishes in 5 ml amounts. The petri dishes were then incubated at 37°C in a humidified atmosphere of 5% CO₂ for two to three days until a confluent monolayer was formed in each plate.

b) Vero cell monolayers: Vero cells⁵ were obtained from W. Stackiw, Manitoba Provincial Virology Laboratory and kept frozen in one ml ampoules

4 Dr. A. M. Wallbank, personal communication.

5 Vero cells are originally derived from the kidney epithelium of an African green monkey (Cercopethicus aethiops).

at -87°C . Monolayers were obtained from the contents of frozen ampoules and sub-cultured every three or four days. Monolayers in small Povitsky bottles⁶ were treated with about 25 ml of a mixture of 1:5,000 EDTA plus 0.02% trypsin in PBS (pH 7.3) for five to ten minutes at room temperature, centrifuged at 160 X g for seven minutes and the cell sediment was resuspended in growth medium at a concentration of 2×10^5 cells/ml. Five ml of this suspension was inoculated into each 60 mm petri dishes which were incubated at 37°C in a humidified atmosphere of 5% CO_2 . Invariably confluent monolayers were formed after 48 hours incubation.

c) L cell monolayers: Strain L-M was obtained from American Type Culture Collection (Certified Cell Line 1.2).

Monolayers were prepared following the same procedure as in Vero except that cells were resuspended in BPRIF⁷ at a concentration of 1.2×10^5 cells/ml and five ml of this was inoculated per 60 mm petri dish.

d) Baby hamster kidney cell monolayers (BHK): BHK-21 was supplied by Microbiological Associates. Monolayers were prepared following the procedure used for Vero cells.

ACCESSORY FACTOR (AF)⁸

The serum containing AF was filtered through a $0.3 \mu\text{m}$ Millipore membrane, sterility tested and stored at -87°C .

6 Kimble glass, two litre capacity.

7 See Appendices A and B.

8 AF serum was kindly supplied by Dr. Kenneth Walls, Communicable Disease Centre, Atlanta, Georgia. (vide Appendix K).

COMPLEMENT

Guinea pig serum⁹ was diluted in PBS-A to contain 1) 25 C'H50/ml and 2) 4 C'H50/ml. It was distributed in two to five ml amounts in sterile stoppered glass tubes in the Revco at -87°C after filtration through $0.3\mu\text{m}$ Millipore membranes. Prior to use, the tubes were immersed in a water bath at 37°C and thawed quickly.

PRESERVATION OF TOXOPLASMA IN LIQUID NITROGEN REFRIGERATOR

1) To an amount of extracellular fluid from L cell culture in BPRIF medium containing live toxoplasma, an equal volume of freeze medium comprising RIF 6.5 ml, calf serum 1.5 ml and DMSO 2.0 ml was slowly added with constant shaking, to give a final concentration of 10% DMSO and 7.5% calf serum. The suspension was distributed in 1.0 ml amounts in sterile glass ampoules which were heat-sealed with a dual-arm, gas-oxygen torch. The sealed ampoules were kept in a cardboard box in a freezer at -87°C overnight and then transferred to the vapour phase of a liquid nitrogen refrigerator (-170°C).

2) Peritoneal exudate from mice infected 72 hours earlier was collected in RIF and, to 3.0 ml of this suspension containing toxoplasma 1.0 ml of calf serum and 1.0 ml of DMSO were slowly added and well mixed to obtain a final concentration of 20% DMSO and about 20% calf serum with a concentration of 3×10^7 toxoplasma/ml. This mixture was distributed in one ml amounts in sterile glass ampoules which were similarly treated as in number 1 above, before being transferred to liquid nitrogen chamber.

⁹ Obtained from Mr. W. Stackiw, Manitoba Provincial Virology Laboratory, where the complement was titrated by the method of Delaat (1964).

3) Peritoneal exudate from mice infected 48 hours earlier was collected in HBSS. Count of toxoplasma and plaque assay of the suspension carried out revealed that it contained 6×10^6 toxoplasma/ml and 2.2×10^6 PFU/ml respectively. Equal volumes of the suspension and each of the 12 freezing mixtures outlined in Table I, in double strength was separately mixed and the final mixture was distributed in 1.0 ml amounts in sterile glass ampoules, heat-sealed, slowly cooled and finally preserved.

PLAQUE ASSAY

Plastic petri dishes¹⁰ 60 by 15 mm were used in all the plaque assay and neutralization by plaque assay experiments. The dishes were seeded with five ml growth medium containing approximately 2×10^5 Vero cells/ml. In experiments where other cells were used, their concentrations are given separately. The cells were allowed to form a confluent monolayer at 37°C in a humidified atmosphere of 5% CO₂ in air, which usually took about 48 hours, when the growth medium was removed and the monolayers were each inoculated with 0.5 ml of toxoplasma suspension of appropriate dilution. When one ml of toxoplasma was used as the inoculum, it was specifically stated under the experiment. Unless stated otherwise, the petri dishes were incubated at 37°C for three hours to allow adsorption of toxoplasma, at the end of which period, the monolayers were overlaid with five ml agar overlay either directly or after removal of inoculum and washing with PBS-A. After the agar overlay had solidified at room temperature (21 - 23°C), the cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. A second agar overlay of five ml was added to each plate on the 6th

¹⁰ Falcon Plastics, a division of Becton, Dickinson & Co.

TABLE I

FINAL CONCENTRATION OF PROTECTIVE SOLUTES

Percent of dimethyl sulfoxide	Percent of glycerol	Percent of dimethyl sulfoxide & glycerol
15	15	7.5 + 7.5
10	10	5.0 + 5.0
5	5	2.5 + 2.5
2.5	2.5	1.25 + 1.25

Composition of freeze mixture is RIF = 5.5 ml, inactivated calf serum = 1.5 ml,
DMSO, glycerol or DMSO and glycerol as given in the Table above and HBSS q.s. to 10 ml.

day and further incubated after allowing it to solidify. On the 11th day three ml neutral red agar was applied to each plate, allowed to solidify at room temperature, incubated for 24 hours and the plaques were read on the 12th day. Plaques were distinct by this time. However, when the plates were allowed to stand at room temperature after incubation for 24 hours, plaques became even more distinct in about six hours and remained thus for another 48 to 72 hours before finally fading away.

NEUTRALIZATION TECHNIQUE

The neutralization technique employed in the study was essentially based on that described by Dulbecco et al. (1956), McBride (1959) and by Ashe and Scherp (1963). Antitoxoplasma serum, whose titre had previously been determined by indirect fluorescent antibody test, was distributed in 0.5 ml amounts in sterile stoppered glass tubes. Throughout the experiment toxoplasma was obtained from mice infected 48 hours earlier and the toxoplasma suspension diluted to contain a known number of live toxoplasma (usually 2×10^6 /ml) as determined by count in haemocytometer after staining with alkaline methylene blue. Toxoplasma suspension was taken in 0.5 ml amounts in separate, sterile, stoppered glass tubes. Whenever complement or accessory factor was used, it was added in 1.0 ml amounts to 0.5 ml of toxoplasma suspension. In some experiments an additional 0.5 ml of suspending fluid was added to make the total volume of antiserum-parasite mixture to 2.5 ml when both were mixed. The suspending or collecting fluid used varied in different experiments as shown under each experiment. Five minutes before mixing, the tubes were kept separately in a water bath at 37°C and thus pre-warmed

to 37°C. At time zero, 0.5 ml of antiserum was aspirated with a two ml pipette and added to the tube containing toxoplasma suspension and was well mixed. At time zero and at appropriate time intervals thereafter (as given under each experiment), 0.5 ml of the mixture was pipetted into 100 ml of the diluent kept at room temperature in order to prevent further neutralization, the antiserum having been diluted 200-fold. The diluent was either PBS-A with two per cent calf serum or HBBS. Of this diluted sample 0.5 ml was assayed as such, and in some experiments as such, as well as after 1-2 or 1-5 further dilutions, according to the expected amount of surviving toxoplasma on two replicate Vero monolayers, allowing three hours adsorption time before the addition of 5.0 ml of nutrient agar overlay per plate.

Control tubes contained instead of antiserum, 0.5 ml of either corresponding preinoculation serum or normal serum (known to have been negative in the IFAT).

In experiments designed to study the antibody effect on toxoplasma in the absence of complement or accessory factor serum, one ml of suspending fluid was added in place of complement or accessory factor serum, to toxoplasma suspension so that the total volume of the suspension and the concentration of toxoplasma remained the same as in tubes with complement or accessory factor serum.

In experiments designed to determine if 1:200 dilution of the mixture and/or the antibody excess had any effect on neutralization, the toxoplasma suspension was initially further diluted to contain 1×10^4 /ml instead of 2×10^6 /ml of toxoplasma, and 0.5 ml of this suspension was added to each tube. This was the only change in the reaction system and

the surviving toxoplasma was assayed at appropriate time intervals directly from the mixture to the monolayers without carrying out 1:200 dilution.

In all the experiments, a second overlay of five ml per plate was added on the 6th day, three ml neutral red agar per plate on the 11th day and the plates were read on the 12th day as detailed under plaque assay. In the initial neutralization experiment an exception was made, in that the amounts of toxoplasma suspension, the buffer and the antiserum or pre-inoculation serum, were twice those in other experiments, namely, 1.0 ml, 3.0 ml and 1.0 ml respectively, in the reaction system, and the dilution was 400-fold, that is, 0.5 ml of the neutralization mixture was blown into 200 ml of diluent.

When statistical analyses were done to test for difference in neutralization with different sera involved, contrasts of the decrease in counts after a time lapse were divided by an estimate of their standard errors. In estimating the standard errors the within cell estimates of variances were pooled in the manner of a two way analysis of variance. A statistic computed as above follows a t-distribution.

EXPERIMENTAL PROCEDURES AND RESULTS

EXPERIMENTAL PROCEDURES AND RESULTS

I. GROWTH CHARACTERISTICS OF TOXOPLASMA IN CELL CULTURES

Growth of Toxoplasma in L cells

Experiment 1. One L monolayer in a 100 mm petri dish was inoculated with 3×10^8 toxoplasma obtained from pooled extracellular fluid from infected cultures and intracellular toxoplasma after trypsinization of two L monolayers in BPRIF medium followed by disruption of cells by forcing through a 26 gauge needle against the inner wall of a glass tube several times. The input multiplicity (IM) was 100:1. After allowing 3 hour's time for adsorption of toxoplasma, 15 ml of BPRIF was added and the plate was incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air.

Growth medium was replaced with fresh BPRIF in 15 ml amounts on days 12, 18 and 22. The count of extracellular, live toxoplasma made at various intervals during the 23 days of its growth, after which the experiment was discontinued, is shown in Figure 1.

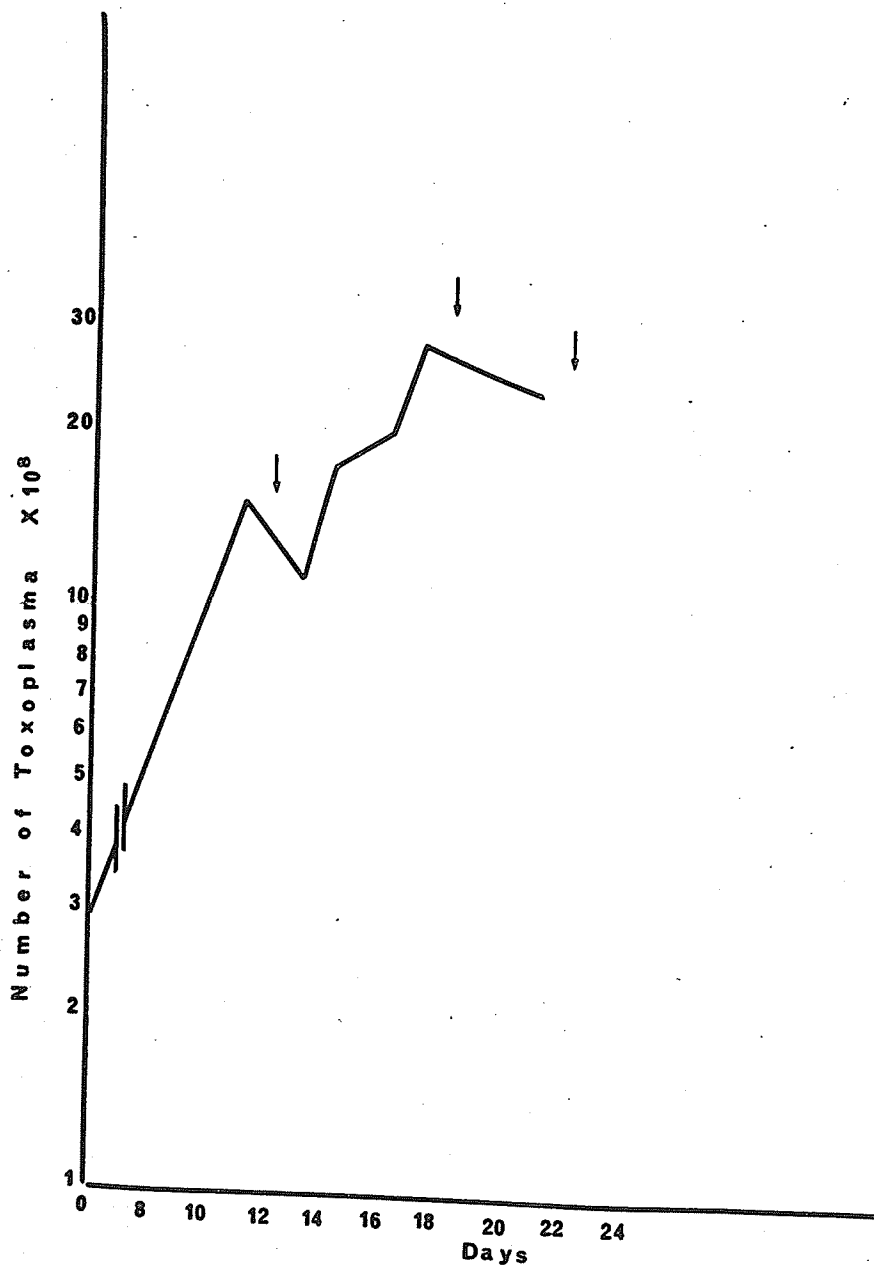
Experiment 2. One 100 mm petri dish containing L monolayer was inoculated with the contents of one frozen ampoule after quickly thawing in water bath at 37°C . The ampoule contained about 6×10^7 toxoplasma in 1.0 ml. (equal parts of freeze medium and extracellular fluid from a L cell culture containing toxoplasma) at the time of preservation in liquid nitrogen in 10% DMSO about a month earlier. In a separate plaque assay (Vide Experiment 6) it was found that the contents of the ampoule were 20 PFU. From the plaquing efficiency of about 1% that was obtained for toxoplasma from mice infected 72 hours earlier (Vide Experiment 8) it could be estimated that the inoculum contained about 2×10^3 toxoplasma, giving an IM = 0.001:1.

Figure 1. Count of extracellular toxoplasma in L monolayer inoculated with 3×10^8 toxoplasma on day 0, at an IM - 100.0.

The number of live toxoplasma in the extracellular fluid is plotted against time in days.

↓ indicates the days when medium was replaced.

FIGURE 1



As in the above experiment, 15 ml. BPRIF was added and the growth was observed for 30 days. At intervals of a few days, count of extracellular toxoplasma was made and the results are presented in Figure 2.

Experiment 3. In all 12-60 mm petri dishes containing L monolayers were used. Two monolayers were trypsinized and the cell-count made under haemocytometer revealed that each monolayer contained 1×10^6 cells. Two plates were used as control and 2 plates each were inoculated with 1×10^6 , 1×10^5 , 1×10^4 and 1×10^3 toxoplasma in 1.0 ml. BPRIF giving an IM of 1.0, 0.1, 0.01 and 0.001 respectively. Toxoplasma was obtained from mice infected 72 hours earlier, collected in HBSS with 10% calf serum and washed once with BPRIF. After adsorption of toxoplasma as in experiment 1, 5 ml. of BPRIF was added to each plate and incubated for 10 days. Extracellular, live toxoplasma counts made on days 8, 9 and 10 are given in Figure 3. Detachment of cells in the two control plates were complete on days 6 and 7.

Experiment 4. Growth of toxoplasma in Vero monolayers.

A prescription bottle¹¹ containing a confluent Vero monolayer was inoculated with 1×10^8 toxoplasma at an IM = 7.0 in 2 ml. of extracellular fluid from a CEF culture infected with toxoplasma and grown in RIF with lamb serum. After allowing an adsorption time of 3 hours, 30 ml. of RIF was added and growth was studied under

11. Dominion glass, capacity 8 ounces.

Figure 2. Count of extracellular toxoplasma in L monolayer inoculated at an IM - 0.001 on day 0.

The number of live toxoplasma in extracellular fluid is plotted against time in days.

↓ shows the days when medium was replaced.

FIGURE 2

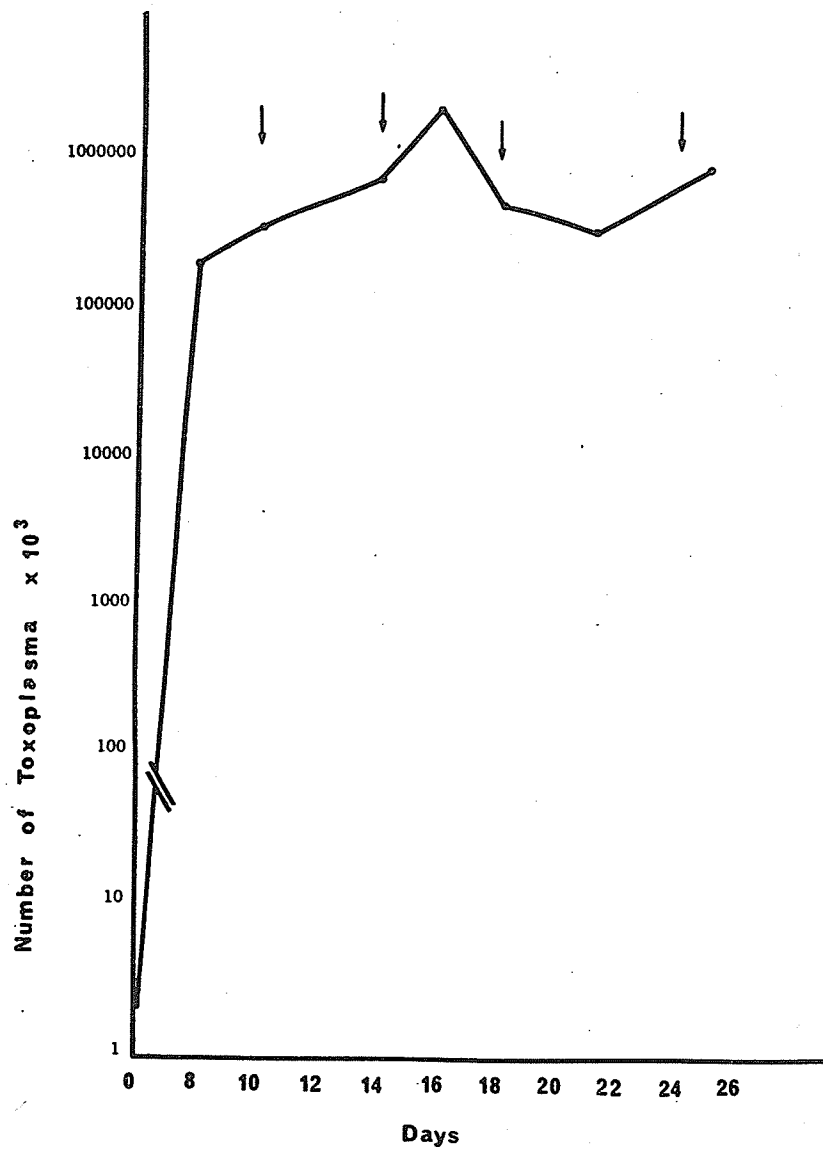
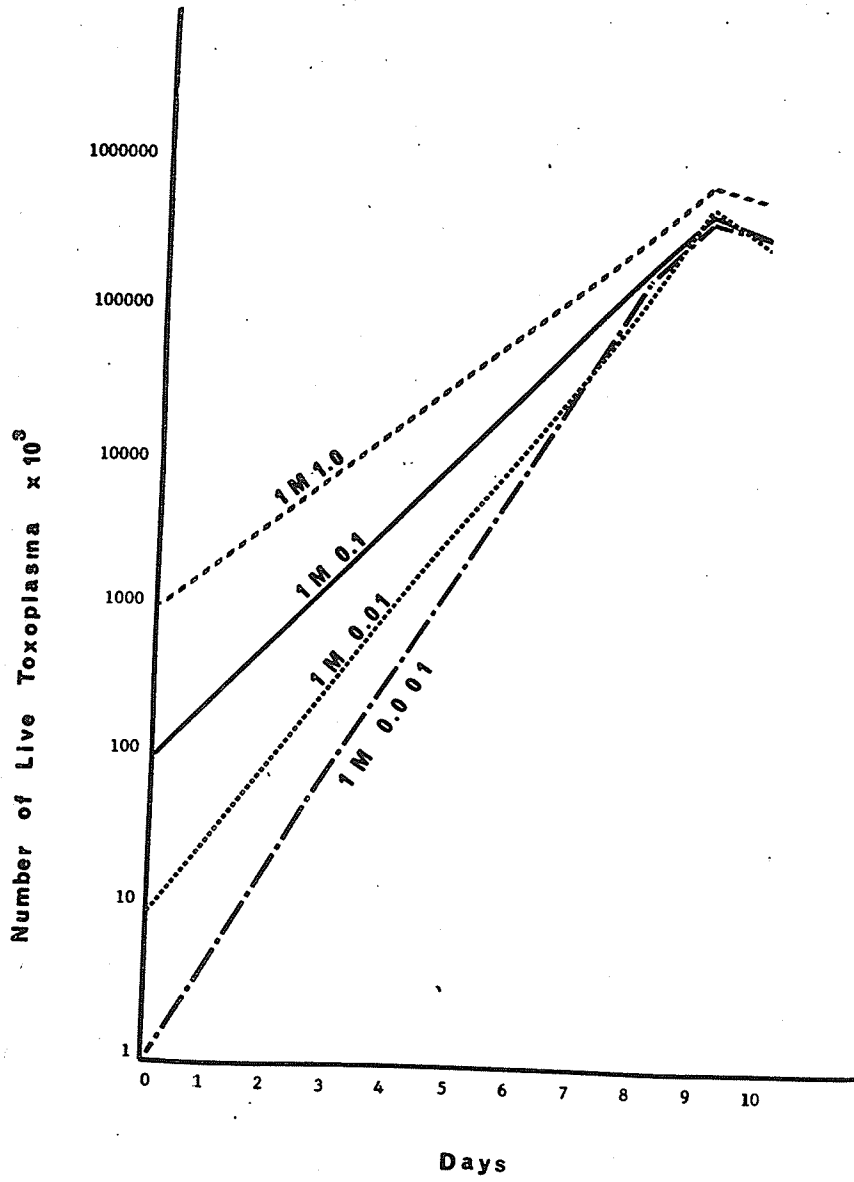


Figure 3. Count of extracellular toxoplasma in four pairs of L monolayers, each pair inoculated at an IM = 1, 0.1, 0.01, and 0.001.

The number of live toxoplasma in the extracellular fluid is plotted against time in days.

FIGURE 3



incubation at 37°C as in Experiment 1. Count of extracellular toxoplasma made at various periods during the 16 days of growth study can be seen in Figure 4. On the 17th day there was complete degeneration of the cell monolayer.

Experiment 5. Growth of toxoplasma in secondary chick embryo fibroblasts.

Secondary CEF monolayers in two T-flasks¹² were each inoculated with 5×10^7 toxoplasma in 1.0 ml of extracellular fluid from L cells with BPRIF. After three hours for adsorption, 5 ml of RIF with lamb serum was added into each flask and growth was allowed at 37°C.

Results of count of extracellular, live toxoplasma as obtained on days 12, 14, 17 and on day 21, when most of the cells showed signs of degeneration, are presented in Figure 5.

In another experiment, secondary CEF in a Blake bottle¹³ was inoculated with 5×10^8 toxoplasma in 2.0 ml of extracellular fluid from a CEF in RIF with lamb serum, at an IM = 10.0, allowed to adsorb and allowed to grow in 75 ml RIF with lamb serum at 37°C. The growth lasted 24 days when the cells were seen degenerating.

There are no reports where comparative studies of growth of toxoplasma in different cell monolayers have been carried out. Even here, there was no controlled, critical study. It was an exercise in tissue culture and it is interesting that it allows certain generalizations to be deduced. Taking into consideration the input multiplicity of infection

12. Falcon Plastics, a division of Becton, Dickinson & Co., capacity 30 ml.

13. Kimble glass, capacity 1,000 ml.

Figure 4. Count of extracellular toxoplasma in Vero monolayers inoculated with 1×10^8 toxoplasma at an $IM = 7.0$.

The number of live toxoplasma in extracellular fluid is plotted against time in days.



indicates the days when medium was replaced.

FIGURE 4

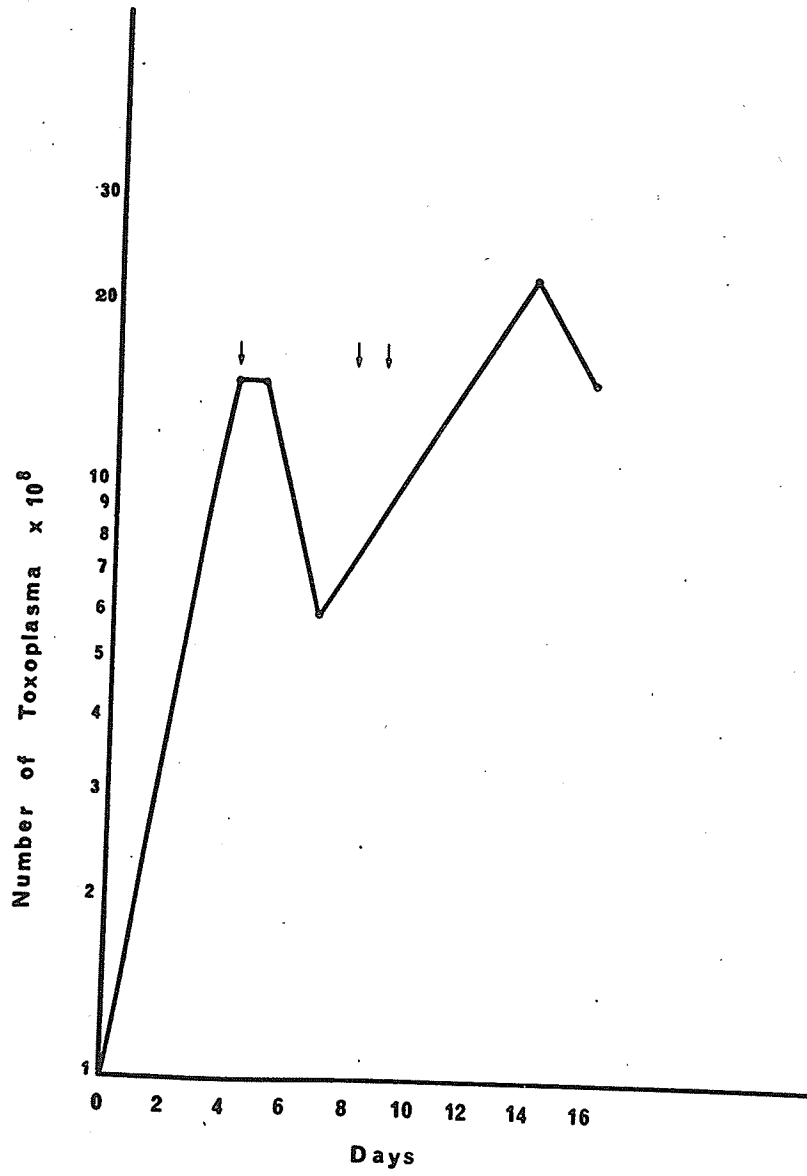
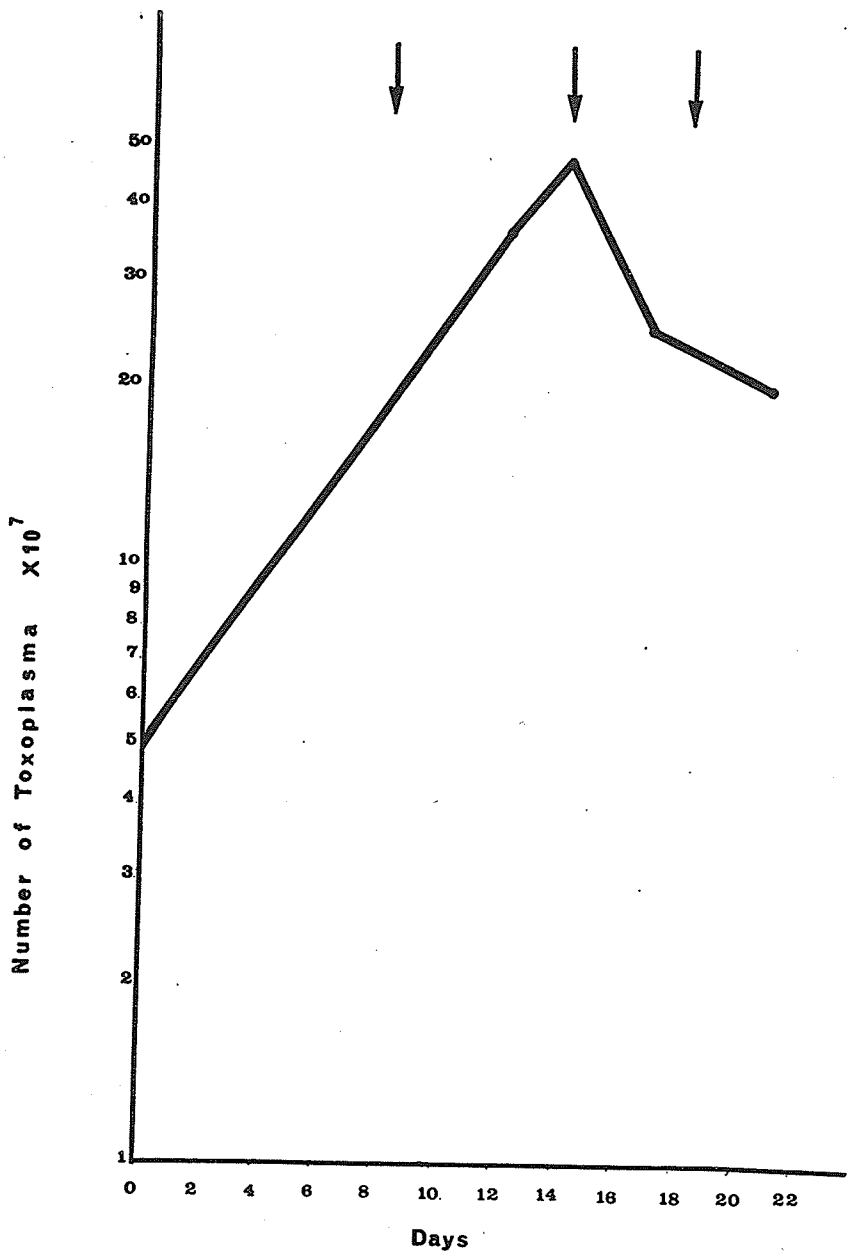


Figure 5. Count of extracellular toxoplasma in secondary CEF monolayers inoculated with 5×10^7 toxoplasma at an IM = 25.0 on day 0.

The number of live toxoplasma in extracellular fluid is plotted against time in days.

↓ indicates the days when medium was replaced.

FIGURE 5



In Vero, secondary CEF and L cells, it is apparent that Vero cells are probably more sensitive to T. gondii than secondary CEF and L cells, in view of the complete degeneration of Vero monolayers in the shortest period of 17 days under study.

In L cells, in both the experiments 1 and 2, with a rather wide range in the input multiplicity, a chronic state of infection was apparent and it looked as if the infection would have persisted for a long time had not the experiments been terminated in about a month's time.

II. PRESERVATION OF T. GONDII IN LIQUID NITROGEN

Apart from the fact that maintenance of many strains of T. gondii by repeated passage in laboratory animals is time consuming and laborious, it is also known that count of live toxoplasma in the haemocytometer under the light microscope is far from satisfactory (Foley and Remington, 1969). It was, therefore, considered worthwhile investigating into the possibility of preservation of toxoplasma in liquid nitrogen and to use a known number of PFU as ascertained by previous plaque assay, in the neutralization experiments to be carried out and thus control the input of toxoplasma with greater accuracy. With this in mind, plaque assay of frozen toxoplasma was carried out in the following two experiments.

(See Discussion)

Experiment 6. Preservation of toxoplasma obtained from mice infected 72 hours earlier, and from tissue culture in liquid nitrogen and their plaque assay.

One ampoule each containing one ml of frozen toxoplasma in liquid nitrogen, obtained 1) from extracellular fluid of L cell culture estimated

to contain at the time of preservation about 6×10^7 toxoplasma in one ml in 10 per cent DMSO and 7.5 per cent calf serum, and 2) from peritoneal exudate of mice infected 72 hours earlier and preserved in 20 per cent DMSO and about 20 per cent calf serum containing a total of about 3×10^7 toxoplasma in 1.0 ml when preserved, was removed from the liquid nitrogen chamber after 137 and 135 days respectively of preservation, and quickly thawed in water bath at 37°C . After thoroughly mixing, the contents of each ampoule was diluted in serial 10-fold dilutions in RIF and each dilution was assayed in two replicate Vero monolayers in 60 mm petri dishes as per the procedure for plaque assay. Results revealed that ampoule 1) contained 20 PFU and ampoule 2) contained 10 PFU representing respectively 6×10^7 and 3×10^7 toxoplasma originally kept in liquid nitrogen.

Experiment 7. Comparative study of the efficacy of preservation in liquid nitrogen of toxoplasma from mice infected 48 hours earlier in various freeze mixtures.

Toxoplasma obtained from mice infected 48 hours earlier and preserved in liquid nitrogen in various freeze mixtures in 1.0 ml amounts in sterile ampoules containing 1.1×10^6 PFU in each ampoule at the time of preservation in liquid nitrogen was assayed after 20 days for the surviving toxoplasma after a dilution of 1-10 in RIF in duplicate Vero cell monolayers. The results are presented in Table II.

Highest number of survivors was obtained in the ampoule preserved in 5% DMSO namely, 4,600 PFU as against 1.1×10^6 PFU of toxo-

TABLE II

PLAQUE ASSAY FOR SURVIVORS OF TOXOPLASMA IN VARIOUS FREEZE
MIXTURE AND PRESERVED IN LIQUID NITROGEN FOR 20 DAYS

Dilution	Percent Dimethyl sulfoxide				Percent glycerol				Percent of Dimethyl sulfoxide + glycerol			
	15%	10%	5%	2.5%	15%	10%	5%	2.5%	7.5+7.5%	5+5%	2.5+2.5%	1.25+1.25%
1-10	* 94	228	460	174	0	0	32	100	2	6	200	0

* Total number of plaques in two plates, each plate inoculated with 0.5 ml of 1-10 dilution.

plasma originally preserved, giving a survival rate of 0.42%.

III. PLAQUE ASSAY OF TOXOPLASMA

Experiment 8. Plaquing efficiency of toxoplasma from mice infected 72 hours earlier, with and without artificial lysis, in Vero monolayers.

Peritoneal exudate from mice infected with T. gondii 72 hours earlier, was collected in RIF containing antibiotics, with sterile glass beads as given under Materials and Methods. The toxoplasma suspension was divided into two equal portions, one of which was forced through a 26 gauge needle against the inner wall of the glass tube several times to liberate intracellular toxoplasma. Count of live toxoplasma in the untreated portion was 5×10^6 /ml and in the artificially lysed portion of suspension, it was 6×10^6 /ml. Three serial 10-fold dilutions of the two portions of suspensions were made in RIF separately and assayed on two Vero monolayers in 60 mm petri dishes, each plate receiving 1.0 ml of the diluted suspension. The plaque counts and the plaquing efficiency of the two suspensions are summarized in Table III. The plaquing efficiency in the artificially lysed suspension was 0.6% as against 1.1% of the untreated portion.

Experiment 9. Plaquing efficiency of toxoplasma from mice infected 48 hours earlier, with and without artificial lysis, in Vero monolayers.

The plaquing efficiency (PE) obtained in the above experiment using "72 hours" toxoplasma in RIF being 1.1% (which is without doubt very low indeed) plaque assay as in Experiment 8 was repeated, this time, however, using exudate containing toxoplasma from mice infected 48 hours earlier. Results presented in Table IV shows a signifi-

TABLE III

PLAQUING EFFICIENCY OF TOXOPLASMA FROM MICE INFECTED 72 HOURS EARLIER, WITH AND WITHOUT ARTIFICIAL LYSIS IN VERO MONOLAYERS

Dilution	Untreated Suspension		Artificially Lysed Suspension	
	Number of Plaques	Plaquing Efficiency	Number of Plaques	Plaquing Efficiency
1-1,000	54 *	1.1%	36 *	0.6%

* Average number of plaques in two replicates.

The undiluted, untreated suspension contained 5×10^6 toxoplasma/ml, and the undiluted artificially lysed suspension contained 6×10^6 toxoplasma/ml.

TABLE IV

PLAQUING EFFICIENCY OF TOXOPLASMA FROM MICE INFECTED 48 HOURS EARLIER, WITH AND WITHOUT ARTIFICIAL LYSIS IN VERO MONOLAYERS

Dilution	Untreated Suspension		Artificially Lysed Suspension	
	Number of Plaques	Plaquing Efficiency	Number of Plaques	Plaquing Efficiency
1-8,000	103 *	33%	152	38%

* Average number of plaques in two replicates.

The undiluted, untreated suspension contained 2.5×10^6 toxoplasma/ml, and the undiluted artificially lysed suspension contained 3.2×10^6 toxoplasma/ml.

cantly higher PE in both the artificially lysed and untreated portions. However, PE of the artificially lysed toxoplasma was higher at 38% than the untreated portion at 33%.

In all the experiments hereafter, unless otherwise stated, toxoplasma from mice infected 48 hours earlier and without artificial lysis was used. Reproducible results were, however, difficult to obtain as the PE varied between 30.0% and 62.1% as shown in Table V when experiments for PE of different batches of untreated toxoplasma from mice infected 48 hours earlier were carried out in various collecting fluids.

Experiment 10. Comparative study of plaque assay of toxoplasma in Vero, L, secondary chick embryo fibroblast and BHK monolayers.

In order to find out which of the cell systems would be most ideal for plaque assay of toxoplasma, plaque assay was carried out using toxoplasma from exudate of mice infected 48 hours earlier and collected in RIF with sterile glass beads. Five serial two-fold dilutions of the suspension were made and each dilution was inoculated in 0.5 ml. amounts into two confluent monolayers of Vero, L, secondary CEF and BHK cells in 60 mm petri dishes. Two agar overlays were added as given under Materials & Methods and the plaques were read on the 12th day.

Results: Plaques obtained in Vero monolayers were distinct, circular in shape, 2 mm in diameter and up to 200 plaques could be easily counted in one monolayer. Plaques started appearing in about 10 hours of incubation at 37°C after the addition of neutral red agar, becoming clearly visible for counting after 24 hours of incu-

TABLE V

PLAQUING EFFICIENCY OF DIFFERENT BATCHES OF TOXOPLASMA FROM MICE INFECTED
48 HOURS EARLIER, SUSPENDED IN VARIOUS COLLECTING FLUIDS WITHOUT
ARTIFICIAL LYSIS

Collecting fluid	Haemocytometer count of Toxoplasma	Number of plaques	Plaquing efficiency in percent
PBS-A	280	114.0 *	40.71
PBS-A with 2% calf serum	210	116 *	55.24
	300	101 *	33.66
	500	190 *	38.00
	210	115.8 +	55.14
RIF	100	30 *	30.00
	300	185.4 **	62.13

* Average number of plaques in two replicates

+ Average number of plaques in five replicates

** Average number of plaques in ten replicates

bation (see Plate 1). Thereafter the plates were removed from the incubator and allowed to stand at room temperature; when in about six hours, the plaques became even more distinct, remaining so for another 48 to 72 hours. Plaques in L monolayers closely resembled those obtained in Vero monolayers in size, shape and number, though they were not as distinct and the plaques appeared in about five hours after the addition of neutral red agar and disappeared in another five hours time. BHK cells presented plaques that appeared larger, about 4 mm in diameter and roughly circular, however, a portion of the cells in the plates were dead. Cell-death was also noticed in CEF monolayers, in which the plaques were very irregular in shape.

Experiment 11. Experiments to test criteria in proof of plaques.

Of the several criteria set forth by Cooper (1967) in proof of plaques, the following were considered important and pertinent in respect of plaques of viruses and were applied to toxoplasma.

1) that plaque count must be proportional to concentration of toxoplasma,

2) that toxoplasma must be regularly isolated from a plaque in higher concentration than from areas away from a plaque,

3) that there should be no plaques in the absence of toxoplasma, in other words, no plaques in the control plates as compared to plaques in test plates, and

4) that toxoplasma must be inhibited by low concentrations of heated specific antiserum and not by preinoculation serum.

Criteria 3 and 4 were satisfactorily met with in experiments pertaining to plaque assay of toxoplasma and neutralization of toxo-

Plate 1. The plaque characteristics of T. gondii (RH) in Vero monolayer as seen on the 12th day after infection.

PLATE 1



plasma by plaque assay respectively¹⁴. Experiments to test criteria 1 and 2 are set out here: -

1) Five serial two-fold dilutions of toxoplasma were made in PBS-A with 2% calf serum and 0.5 ml of each dilution was inoculated into each of a set of 5 Vero monolayers in 60 mm petri dishes and assayed for plaques as described in Materials and Methods for plaque assay.

The results of the experiment are presented in Table VI in which the number of plaques in each plate for each dilution, as also the mean number of plaques and standard deviation for each dilution are given. Figure 6 illustrates that the increase of plaque count is directly proportional to concentration of toxoplasma and approximately follows a linear relationship with dose.

The regression line was originally estimated on untransformed data by a least squares method and the line was drawn. However, due to non-homogeneous variances the least squares line is not the best or minimum variance line. The estimated standard deviations at the four dilutions are 1.14, 1.52, 3.90 and 8.14 while the mean values are 14.4, 32.60, 61.20 and 115.80 respectively. For the original line drawn the Y intercept is very small and it is obvious from looking at the plotted data that in weighting by the inverse of the variances this intercept will be still smaller. For this reason, coupled with the reasonable-

14. As to criterion 4, neutralization of toxoplasma was demonstrated by rabbit, rat and rooster antisera whereas there was no neutralization when the respective preinoculation serum was used, thus showing that neutralization of toxoplasma was specific. However, the dilution of antisera used was either 1-4 or 1-5.

ness of the assumption that the line goes through the origin, the model for the regression line was chosen to be $Y = \beta X^\alpha$. A logarithm transformation gives $\log Y = \log \beta + \alpha \log X$ and the least squares estimate of α is 0.993 which is not significantly different from 1.00 or $Y = \beta X'$. However, a test for linearity of the transformed data does demonstrate a significant deviation from a straight line. This appears to be due to a slight deflection downwards at the high counts. It should be noted that the dispersions of counts at given dilutions are quite small and they do not estimate dilution errors which would account for some of the nonlinearity.

2) To demonstrate that toxoplasma could be obtained from a plaque and not from areas away from a plaque, one plaque was aspirated with a sterile Pasteur pipette from one of the plates with a total of fourteen plaques, collected in 1.0 ml PBS-A with 2 per cent calf serum (diluent), thoroughly mixed and 0.5 ml each was inoculated into two Vero monolayers and assayed for plaques. Similarly areas between two plaques was aspirated, collected in 1.0 ml diluent and similarly assayed separately. Whereas a total of 22 plaques were obtained from toxoplasma from the aspirated plaque, the two plates inoculated with material from an inter-plaque area developed no plaques.

Further, toxoplasma aspirated from another plaque and collected in the diluent as above and stained with alkaline methylene blue dye solution revealed live toxoplasma when examined under the light microscope.

Figure 6. Relationship between plaque numbers and concentration of toxoplasma.

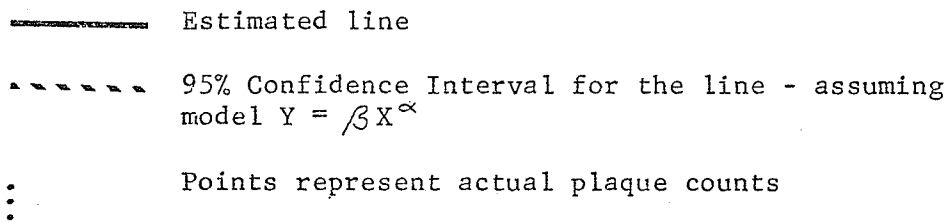


FIGURE 6

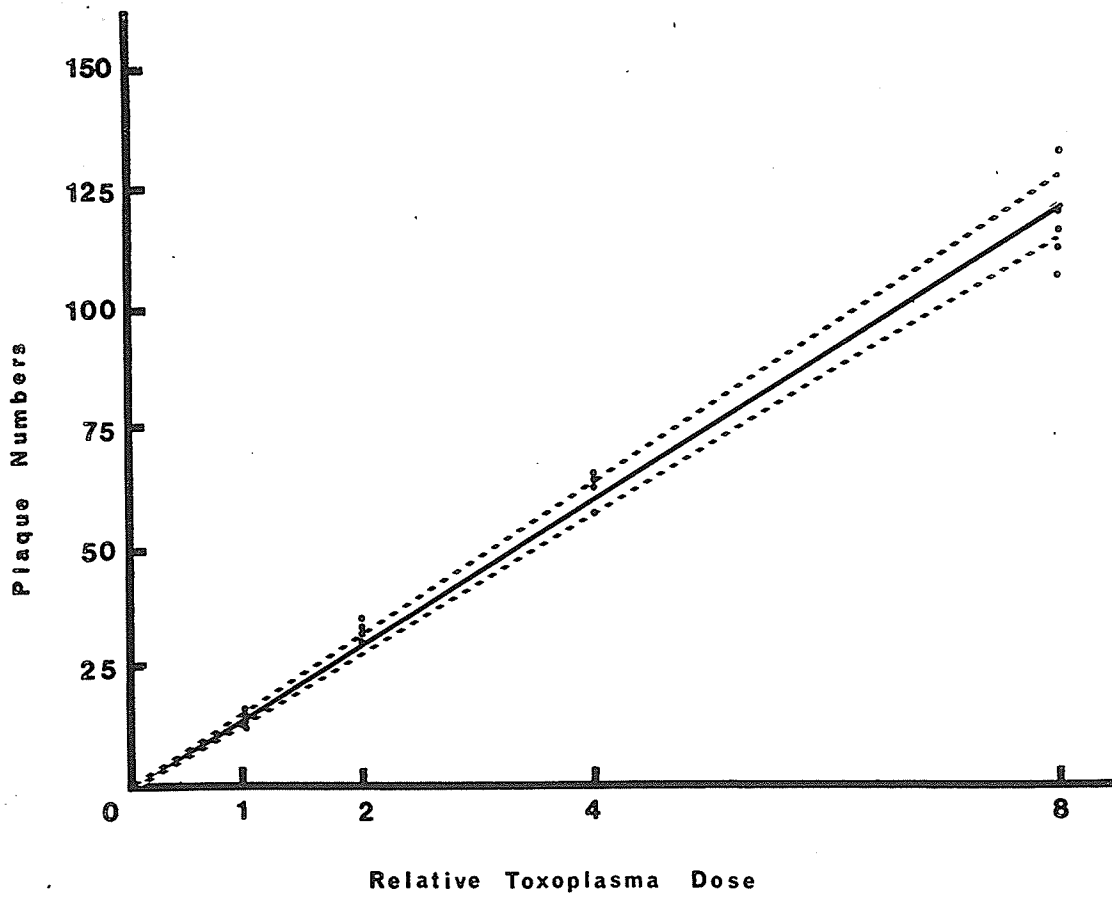


TABLE VI

RELATIONSHIP BETWEEN CONCENTRATION OF TOXOPLASMA AND PLAQUE NUMBERS

Dilutions	Plates					Mean \pm S.D. **
	#1	#2	#3	#4	#5	
Undiluted	TNTC*	TNTC	TNTC	TNTC	TNTC	-
1-2	128	106	115	112	118	115.8 \pm 8.14
1-4	63	64	57	57	65	61.2 \pm 3.89
1-8	32	31	33	32	35	32.6 \pm 1.52
1-16	14	16	13	14	15	14.4 \pm 1.14

* TNTC = Too numerous to count

** S.D. = Standard deviation

Experiment 12. Stability of toxoplasma at 37°C by plaque assay.

1) Toxoplasma from the peritoneal exudate of mice infected 48 hours earlier was collected in PBS-A with 2 per cent calf serum. Further dilution of the suspension was made in the collecting fluid such that the toxoplasma concentration was 6×10^5 /ml. Ten ml of this suspension was taken in a sterile stoppered glass tube which was then placed in a water bath at 37°C, care being taken to see that the level of the water bath was well above the level of the suspension in the glass tube. Samples were drawn at the initiation of the experiment and at intervals of 1 hour, 2 hours, 4 hours, 8 hours and 16 hours. During sampling, the suspension of toxoplasma was thoroughly mixed. From 1.0 ml of the suspension, three serial 10-fold, and three serial two-fold dilutions were obtained using the collecting fluid as diluent. From the last four dilutions, assay for plaques was done on two replicate Vero monolayers for each dilution, using 0.5 ml/plate. The results are presented in Figure 7.

2) A similar experiment was carried out using toxoplasma from peritoneal exudate of mice infected 36 hours earlier and the results are shown in Figure 8.

Toxoplasma from the exudate of mice infected 48 hours were inactivated more readily than the "36 hours" toxoplasma, the former being inactivated more than 2 log units in 8 hours at 37°C when suspended in PBS-A with 2% calf serum, whereas toxoplasma obtained from

Figure 7. Stability of toxoplasma at 37°C in PBS-A with 2% calf serum by plaque assay ("48 hours toxoplasma").

FIGURE 7

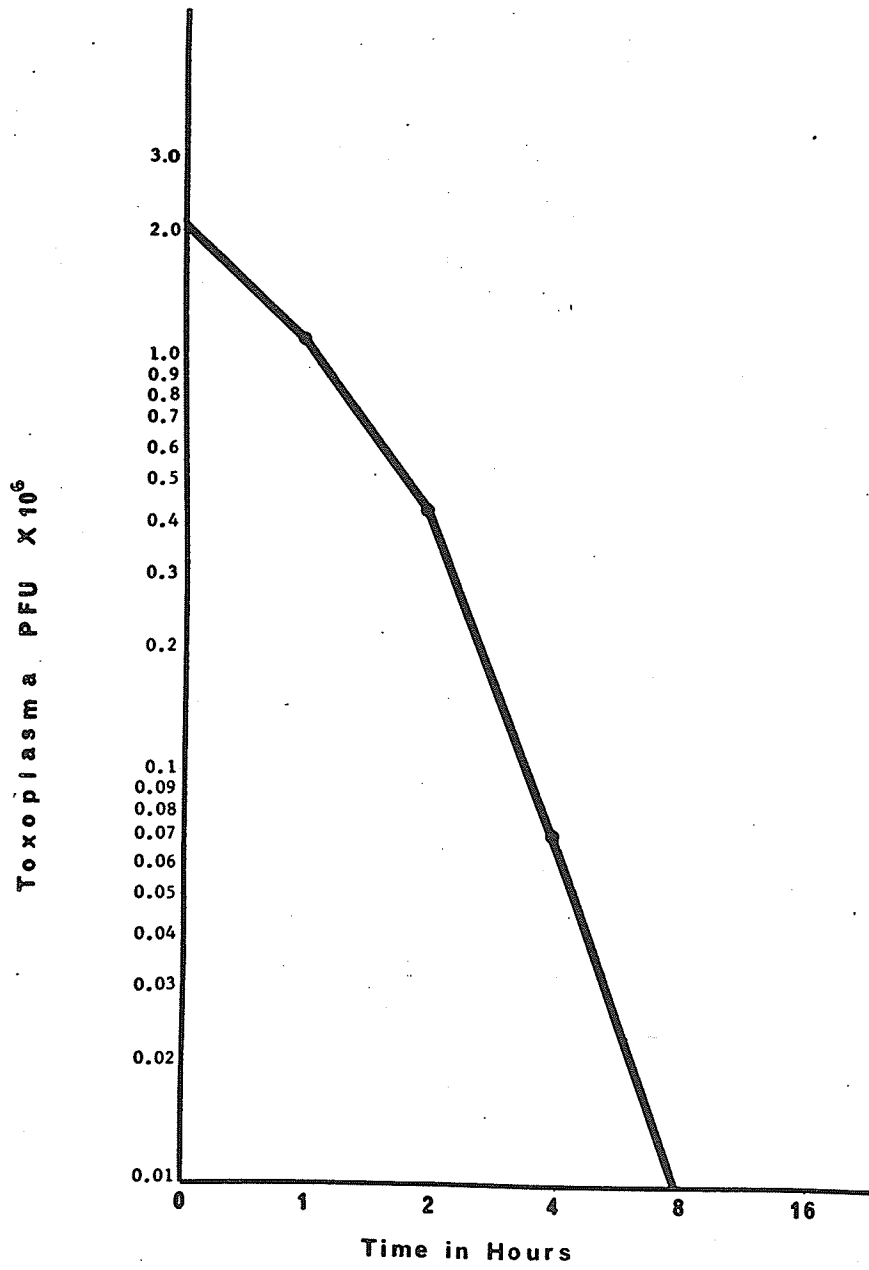
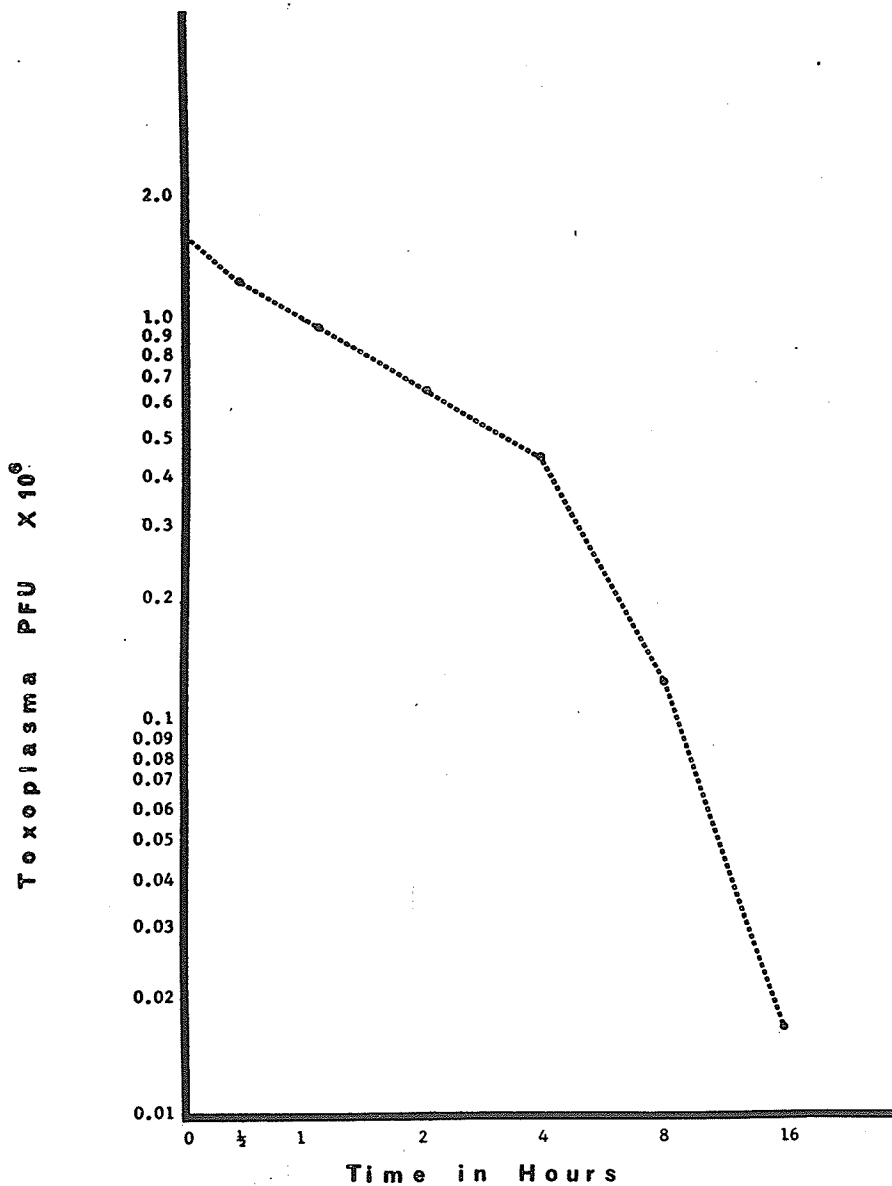


Figure 8. Stability of toxoplasma at 37°C in PBS-A with 2% calf serum by plaque assay ("36 hours toxoplasma").

FIGURE 8



mice infected 36 hours earlier survived over 16 hours.

Inactivation of the parasites was about 1 log unit during the 1st hour and over 2 log units in 8 hours in the 1st experiment with "48 hours" toxoplasma. The longer survival of toxoplasma from mice infected 36 hours earlier should not be surprising due to the fact that the "48 hours" toxoplasma suspension would contain a large proportion of toxoplasma population exposed to extracellular existence for a greater length of time, consequent on which greater degree of degeneration should set in among them than the "36 hours" toxoplasma.

The above result is not in disagreement with the findings of Lycke and Lund (1964b) who were able to obtain survival of toxoplasma over 30 hours at 37^oC, when one considers that tissue culture medium in which they suspended toxoplasma contained 20% human dye-test negative serum as against 2% calf serum used in the present study. However, it must be stressed that the inactivation of toxoplasma as measured by them, namely, by the parasite count and by the number of organisms which passed from the fluid of cultures into the cells as observed under the microscope probably is not as accurate as plaque assay which should give a more accurate indication of the number of virulent toxoplasma present in a suspension.

It is interesting to recall that Chaparas and Schlesinger (1959) studying the survival of toxoplasma in various media at room temperature by plaque assay reported that Hanks' BSS was the least favourable medium in which 99% of the PFU were lost in three hours whereas HBSS containing chick embryo extract in concentrations of 5 to 50% preserved 80 to 100% of PFU for 3 to 4 hours, and 50 to 60%

for 6 hours.

Experiment 13. Stability of toxoplasma at 37°C by parasite count.

Toxoplasma from peritoneal exudate of mice infected 48 hours was collected in 10 ml. of PBS-A with 2% calf serum and, after the count of toxoplasma in the suspension was made, the suspension was kept in water bath at 37°C and the inactivation of toxoplasma was assessed by taking samples at various intervals and counting the live toxoplasma under the microscope after diluting 1:10 in alkaline methylene blue.

The experiment was repeated a second time on a different day and the results of both the experiments as seen in Figure 9 reveal that the stability of toxoplasma varied markedly from one batch of suspension to another (Lycke and Lund 1964b). The rate of inactivation as judged by the number of live toxoplasma that take up the alkaline methylene blue stain is markedly slower than the inactivation rate obtained by plaque assay. A large number of toxoplasma judged as alive by the alkaline methylene blue stain do not produce plaques, that is, do not penetrate cells and multiply. Since the capacity to produce plaques and the capacity to take up alkaline methylene blue stain represent two different properties of toxoplasma, the variation in the results of stability of toxoplasma at 37°C by plaque assay and particle count becomes obvious.

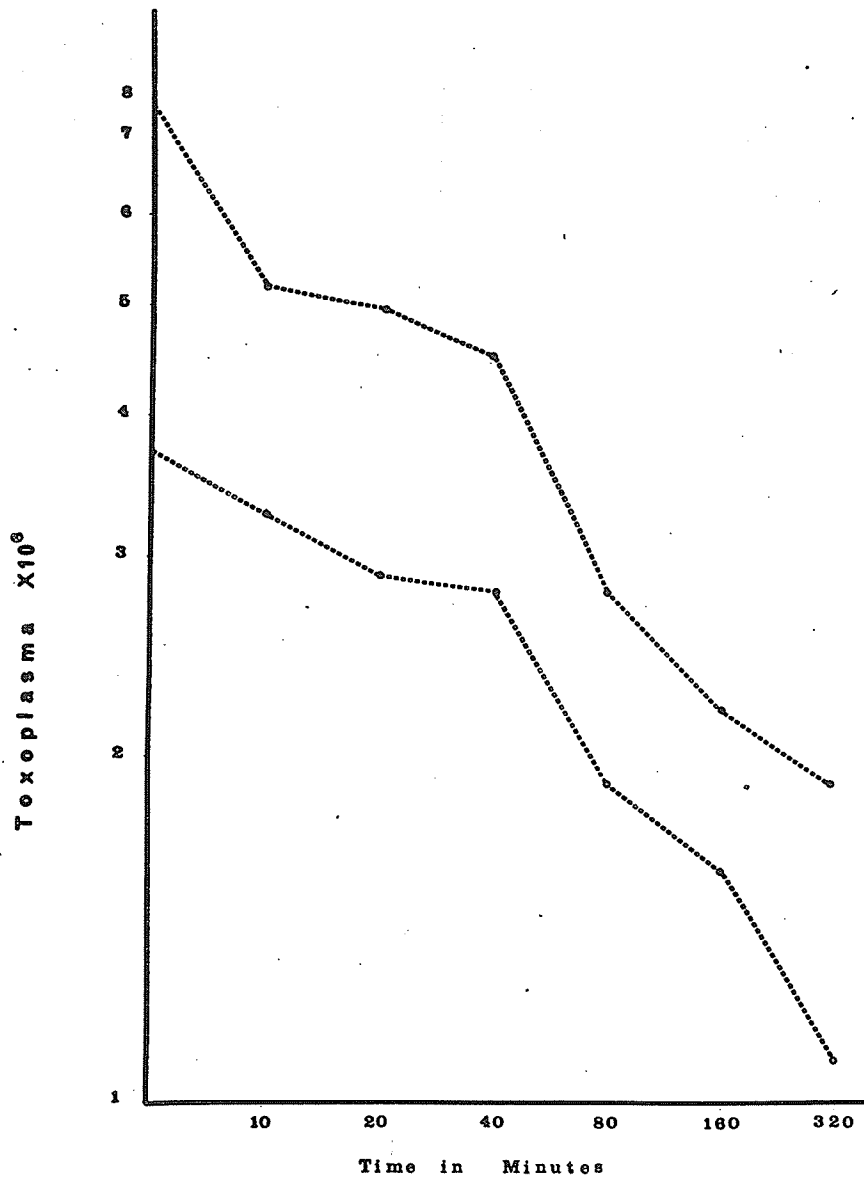
Experiment 14. Stability of toxoplasma at 5°C by plaque assay.

This experiment is similar to Experiment 12 in every respect except that the toxoplasma suspension was kept in the refrigerator at 5°C and the sampling time intervals were longer and ex-

Figure 9. Stability of toxoplasma at 37°C in PBS-A with 2% calf serum by parasite count ("48 hours toxoplasma").

The two lines represent the results obtained in the two experiments in Experiment 13.

FIGURE 9



tended up to 192 hours as can be seen from the results depicted in Figure 10. Complete inactivation of toxoplasma did not take place until 96 hours. From the results of Experiments 12 and 13, it is clear that survival of toxoplasma is many times greater at 5°C than at 37°C.

Experiment 15. Adsorption rate of toxoplasma in Vero monolayers at 37°C.

Toxoplasma suspension collected as in Experiment 12 containing 6×10^6 toxoplasma/ml was serially diluted by four 10-fold, followed by two two-fold dilutions. From the last three dilutions 14 Vero monolayers per dilution were inoculated with 0.5 ml/plate and two plates per dilution were overlaid at 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours and 8 hours after inoculation. The average number of plaques in the plates were multiplied by the dilution factor to obtain the plaques in the stock suspension containing 6×10^6 toxoplasma. Significant increase of adsorption of toxoplasma was observed within the first 30 minutes, later increase being minimal up to 3 to 4 hours. (Figure 11). This is in good agreement with the findings of Chaparas and Schlesinger (1959) in a similar experiment using primary CEF monolayers, where they observed, among other things, that adsorption for 90 to 180 minutes yielded almost identical numbers of plaques, as well as with the findings of the rate of penetration of T. gondii in HeLa cells by Lycke et al. (1964b) as judged by the RNIU under phase contrast microscopy and the number of infective parasites derived therefrom.

IV. NEUTRALIZATION

Experiment 16. Neutralization of toxoplasma by rabbit and rat antisera.

Figure 10. Stability of toxoplasma at 5°C in PBS-A with 2% calf serum by plaque assay ("48 hours toxoplasma").

FIGURE 10

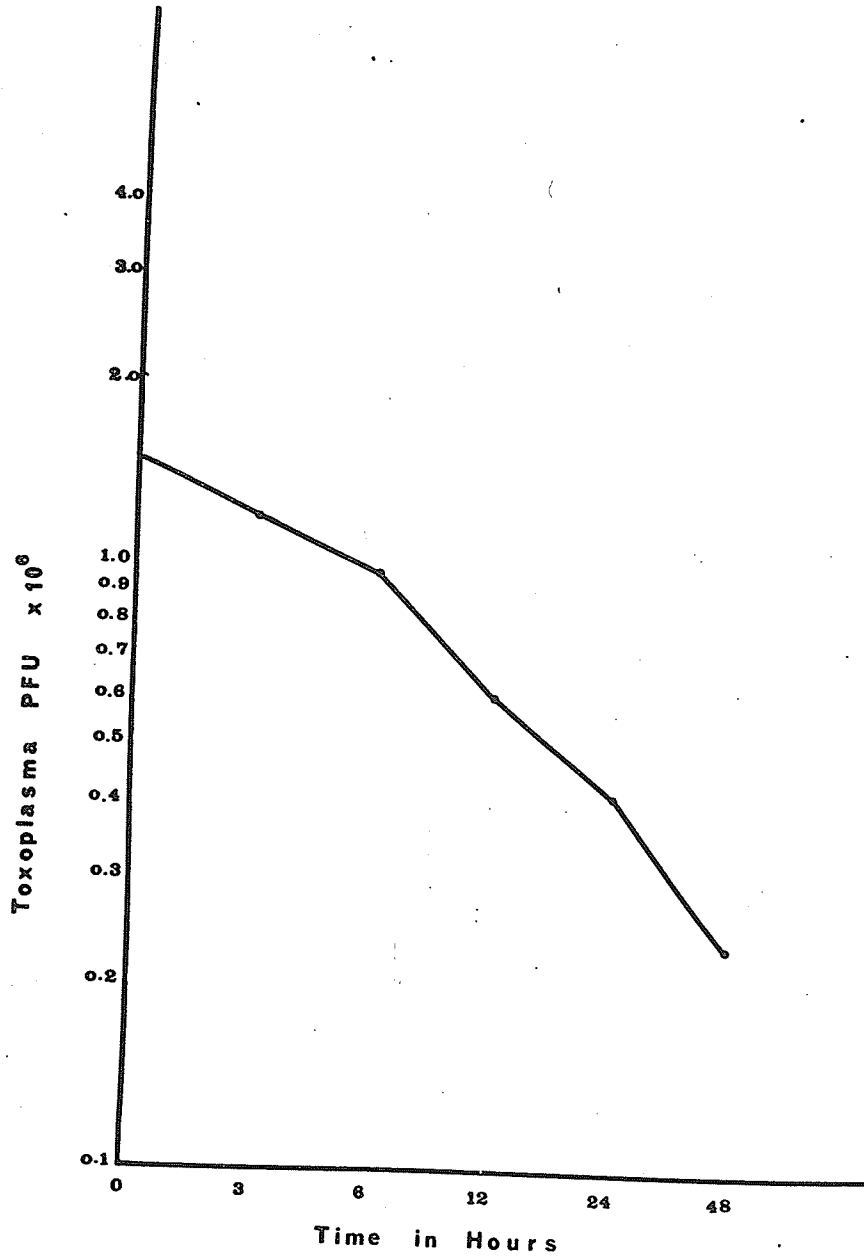
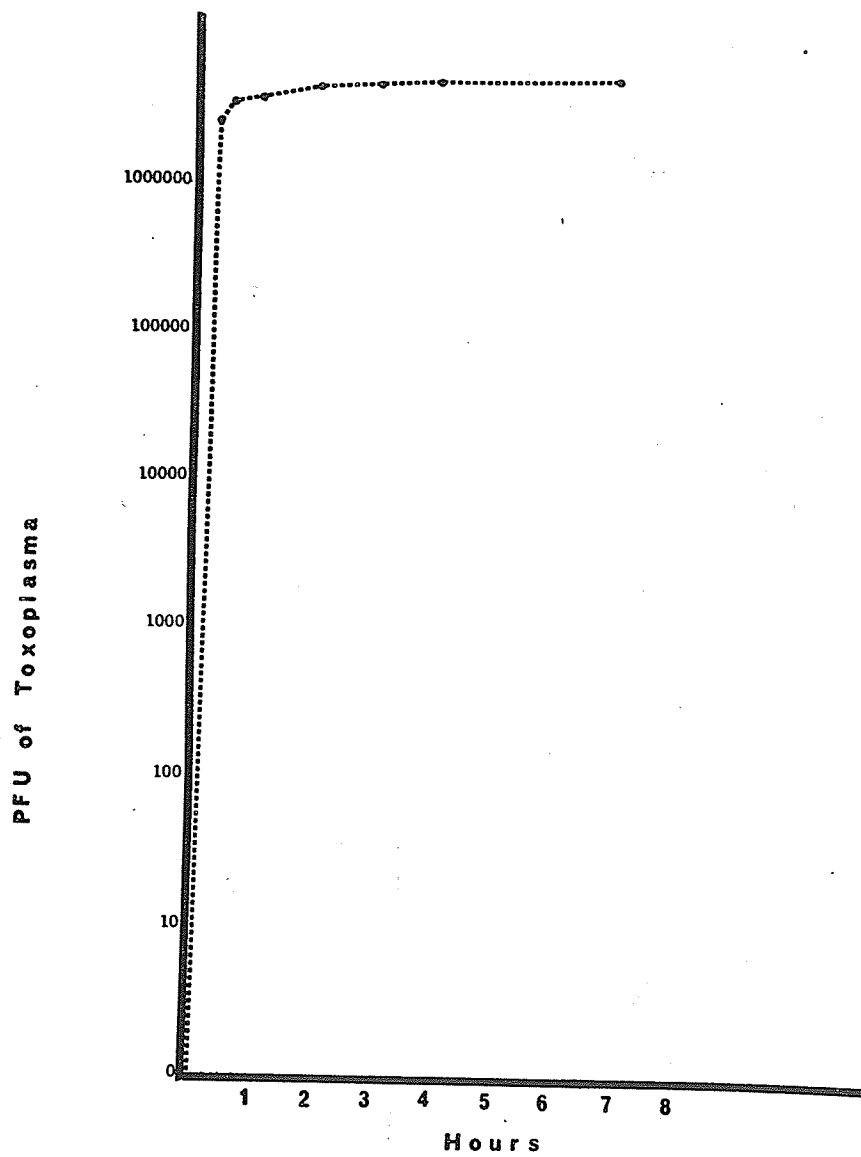


Figure 11. Adsorption rate of toxoplasma at 37°C in Vero monolayers.

FIGURE 11



In the test system, 1 ml of toxoplasma suspension containing 6×10^5 toxoplasma in PBS-A, plus 3 ml of PBS-A with 2% calf serum were taken in stoppered test tube A. In test tube B, 1 ml of rabbit antitoxoplasma serum with a known titre of 1:32 (IFAT) was taken and the contents of the two test tubes were mixed at 0 time, but not before the contents of both the tubes were equilibrated at 37°C , care being taken to see that the level of the water bath was well above the level of the neutralization mixture in the test tube. In the control system, 1 ml of rabbit pre-inoculation serum, previously tested and found negative in IFAT was used in place of rabbit antitoxoplasma serum, in tube B. Tube A contained the same as in tube A of the test system.

At 5 and 20 minute intervals, 0.5 ml of the neutralization mixture, after the mixture was well mixed, from the test and the control tubes was blown separately into stoppered glass bottles containing 200 ml of PBS-A with 2% calf serum in each, at room temperature ($21-23^{\circ}\text{C}$) thus bringing about a dilution of 400-fold. After thoroughly mixing, the diluted samples in 0.5 ml volumes were inoculated onto each of two Vero monolayers for assay. A further set of two Vero monolayers was each inoculated in 0.5 ml volumes after a 2-fold dilution of the above dilution mixtures of both the test and the control.

A similar experiment using rat antitoxoplasma serum with an IFAT titre of 1:128 in the test system and rat pre-inoculation serum with a negative titre in IFAT in the control system was carried out.

The results of the experiment are summarized in Table VII.

There was no neutralization of toxoplasma at 5 minutes and 20 minutes by either rabbit antiserum or rat antiserum.

Experiment 17. Neutralization of toxoplasma by rabbit and rat antisera, with and without complement.

Toxoplasma from mouse exudate collected in HBSS-A was further diluted in HBSS-A to contain 2×10^6 toxoplasma/ml. Test system with complement contained 0.5 ml of the toxoplasma suspension containing 1×10^6 toxoplasma, 1.0 ml of complement containing 25 C'H50 and 0.5 ml of rabbit antitoxoplasma serum having an IFAT titre of 1:128 or rat antitoxoplasma serum with a 1:256 IFAT titre. In the control systems, the antiserum was replaced by the respective rabbit or rat pre-inoculation serum, tested and found negative in IFAT.

In the test and control systems without complement, 1.0 ml of PBS-A was added in place of complement.

After incubation for 1 hour in water-bath at 37° the neutralization mixture in 0.5 ml volumes in each of the test and control systems, with as well as without complement, of rabbit and rat sera, was added to 100 ml diluent (PBS-A with 2% calf serum) contained in stoppered bottles, making a 200-fold dilution, 0.5 ml of the latter being inoculated directly onto each of two replicate monolayers after thorough mixing in the bottle.

The results are outlined in Tables VIII and IX. There was no neutralization of toxoplasma in the absence of complement by either the rabbit and the rat antisera in 1 hour. However, during the same period of neutralization, the rabbit and the rat antisera effected 44.2 and 36.0 per cent neutralization respectively, in the presence

TABLE VII

NEUTRALIZATION OF TOXOPLASMA BY RABBIT AND RAT ANTITOXOPLASMA SERA

Neutralization time	Control Rabbit preinoculation serum, @IFAT-ve +	Rabbit antitoxoplasma serum IFAT 1:32 *	Percent neutralization	Control Rat preinoculation serum, IFAT-ve +	Rat antitoxoplasma serum, IFAT 1:128 *	Percent neutralization
5 minutes	105 ‡	104	0.95	104	107	0
20 minutes	86	90	0	94	102	0

* Test system comprised 1 ml of suspension containing 6×10^5 toxoplasma in PBS-A + 3 ml PBS-A with 2% calf serum + 1.0 ml rabbit or rat antitoxoplasma serum.

+ Control system contained 1 ml of rabbit or rat preinoculation serum instead of antiserum; otherwise same as test system.

‡ Average number of plaques in two replicates.

@ IFAT - indirect fluorescent antibody test.

TABLE VIII

NEUTRALIZATION OF TOXOPLASMA BY RABBIT ANTISERUM, WITH AND WITHOUT COMPLEMENT

Neutralization time	<u>With complement</u>		Percent neutralization	<u>Without complement</u>		Percent neutralization
	Control Rabbit preinoculation serum, @IFAT-ve +	Rabbit antitoxoplasma serum, IFAT 1:128 *		Control Rabbit preinoculation serum, IFAT-ve +	Rabbit antitoxoplasma serum, IFAT 1:128 **	
1 hour	86 †	48	44.2	50	52	0

@ IFAT - indirect fluorescent antibody test.

+ Control system contained 0.5 ml rabbit preinoculation serum instead of rabbit antiserum; otherwise same as test system.

* Test system consisted of 0.5 ml suspension containing 1 million toxoplasma in HBSS-A + 1.0 ml complement containing 25 C'H50 + 0.5 ml rabbit antitoxoplasma serum.

** The ratio of toxoplasma suspension: buffer: antiserum = 1:2:1.

† Average number of plaques in two replicates.

TABLE IX

NEUTRALIZATION OF TOXOPLASMA BY RAT ANTISERUM, WITH AND WITHOUT COMPLEMENT

Neutralization time	<u>With complement</u>		Percent Neutralization	<u>Without complement</u>		Percent Neutralization
	Control Rat preinoculation serum, @IFAT-ve +	Rat antitoxoplasma serum, IFAT 1:256 *		Control Rat preinoculation serum, IFAT-ve +	Rat antitoxoplasma serum, IFAT 1:256 **	
1 hour	50 †	32	36.0	26	27	0

@ IFAT - indirect fluorescent antibody test.

+ Control system contained 0.5 ml rat preinoculation serum instead of rat antiserum, otherwise same as test system.

* Test system consisted of 0.5 ml suspension containing 1 million toxoplasma in HBSS-A + 1.0 ml complement containing 25 C'H50 + 0.5 ml rat antitoxoplasma serum.

** The ratio of toxoplasma suspension: buffer : antiserum = 1:2:1.

† Average number of plaques in two replicates.

of 25 C'H50 complement when compared with their respective controls.

Experiment 18. Neutralization of toxoplasma by rabbit antiserum in the presence of complement, inactivated complement and without complement.

HBSS was used as collecting fluid to suspend toxoplasma contained in the mouse exudate, as diluent and also as buffer.

In the three test systems, the ratio of toxoplasma suspension, complement or inactivated complement or buffer, and antiserum = 1:2:1; 1.0 ml of complement contained 4 C'H50. In each test and control system, 0.5 ml of toxoplasma suspension containing 1×10^6 toxoplasma was used. In the control systems, rabbit pre-inoculation serum in 0.5 ml volumes was taken in place of antiserum used in test systems.

The neutralization mixture, after 200-fold dilution in HBSS, was assayed on two replicate monolayer plates inoculated with 0.5 ml amounts onto each at 0 time, that is, immediately after mixing the toxoplasma suspension + complement or inactivated complement or buffer, and antiserum or pre-inoculation serum, and after 1 hour.

Results documented in Table X¹⁵ shows that maximum neutralization by rabbit antiserum was evidenced in the presence of inactivated complement, namely 36.9% compared to 21.1% neutralization in the presence of complement and no significant neutralization in the absence of complement in 1 hour's neutralization.

-
15. Though the difference in neutralization with complement, as opposed to with inactivated complement, is not significant at the 5% level, the p-value for the difference observed is less than 0.15.

TABLE X

NEUTRALIZATION OF TOXOPLASMA BY RABBIT ANTISERUM IN THE PRESENCE OF COMPLEMENT,
INACTIVATED COMPLEMENT AND WITHOUT COMPLEMENT

Neutralization time	<u>With complement</u>			<u>With inactivated complement</u>			<u>Without complement</u>		
	Control Preinoculation serum +	Antiserum *	Percent neutralization	Control Preinoculation serum +	Antiserum *	Percent neutralization	Control Preinoculation serum +	Antiserum ‡	Percent neutralization
1 hour	204**	161	21.1	241	152	36.9	190	182	4.2

+ Control system contained 0.5 ml of rabbit preinoculation serum, (negative in indirect fluorescent antibody test), instead of rabbit antiserum; otherwise same as test system.

* Test system comprised 0.5 ml suspension containing 1 million toxoplasma in HBSS + 1 ml complement containing 4 C'H50 and 0.5 ml rabbit antitoxoplasma serum, with a titre of 1:128 in indirect fluorescent antibody test.

** Average number of plaques in two replicates.

‡ The ratio of toxoplasma suspension : buffer : antiserum = 1:2:1.

Experiment 19. Neutralization of toxoplasma by rooster antiserum, with and without complement.

This experiment is identical to the previous experiment 18 in all details except that rooster antitoxoplasma serum (IFAT 1:64) was tested for neutralization and PBS-A with 2% calf serum was used as collecting fluid, buffer and as diluent.

From the results outlined in Table XI it can be seen that neutralization without complement was markedly greater than the neutralization in the presence of complement, namely, 51.9% and 37.9% respectively.

In a separate but similar experiment in which toxoplasma was collected in normal saline solution instead of PBS-A with 2% calf serum, similar results were obtained as shown in Table XII. However, neutralization at 1 hour in the absence of complement was nearly twice the neutralization obtained in the presence of complement¹⁶. At 0 time, 16.6% neutralization was obtained in the absence of complement (Vide Discussion).

Experiment 20. Neutralization of toxoplasma by rabbit and rooster antisera, with and without accessory factor serum.

In the test systems with accessory factor (AF) serum, 0.5 ml of toxoplasma suspension containing 1×10^6 toxoplasma, 1.0 ml of AF serum and 0.5 ml of rabbit or rooster antitoxoplasma serum were taken. In place of AF serum in test systems without AF serum, 1.0 ml of buffer was used.

16. The difference in neutralization with and without complement is significant at $\alpha = 0.10$ $0.05 < p < 0.10$

TABLE XI

NEUTRALIZATION OF TOXOPLASMA BY ROOSTER ANTISERUM, WITH AND WITHOUT COMPLEMENT

Neutralization time	<u>With complement</u>		Percent Neutralization	<u>Without complement</u>		Percent Neutralization
	Control Normal serum +	Antiserum *		Control Normal serum +	Antiserum †	
1 hour	29 **	18	37.9	54	26	51.9

+ Control system contained 0.5 ml normal rooster serum, (negative in indirect fluorescent antibody test), instead of antiserum; otherwise same as test system.

* Test system comprised 0.5 ml suspension containing 1 million toxoplasma in PBS-A with 2% calf serum + 0.5 ml PBS-A with 2% calf serum (buffer) + 1.0 ml complement containing 4 C'H50 and 0.5 ml rooster antitoxoplasma serum with 1:64 indirect fluorescent antibody test titre.

† The ratio of toxoplasma : buffer: antiserum = 1:3:1.

** Average number of plaques in two replicates.

TABLE XII

NEUTRALIZATION OF TOXOPLASMA BY ROOSTER ANTISERUM, WITH AND WITHOUT COMPLEMENT

Neutralization time	<u>With complement</u>		Percent neutralization	<u>Without complement</u>		Percent neutralization
	Control Normal serum +	Antiserum *		Control Normal serum +	Antiserum ‡	
0 time	100 **	106	0	143	120	16.1
1 hour	92	76	17.4	122	81	33.6

+ Control system contained 0.5 ml normal rooster serum, (negative in indirect fluorescent antibody test), instead of antiserum; otherwise same as test system.

* Test system comprised 0.5 ml toxoplasma suspension containing 1 million toxoplasma in normal saline solution + 0.5 ml PBS-A with 2% calf serum, + 1.0 ml complement containing 4 C'H50 and 0.5 ml rooster antitoxoplasma serum with 1:64 indirect fluorescent antibody test titre.

‡ The ratio of toxoplasma suspension : buffer : antiserum = 1:3:1.

** Average number of plaques in two replicates.

In the control systems, respective pre-inoculation sera were used instead of antisera.

The collecting fluid, buffer and diluent was HBSS.

Assay for neutralization was carried out in the usual manner in two replicate monolayers and the results are presented in TABLE XIII. Rabbit antiserum (IFAT 1:128) produced no neutralization at 0 time whether or not AF serum was present, and at 1 hour, 8.9% neutralization was observed in the presence of AF serum but not in its absence¹⁷. Rooster antiserum (IFAT 1:128), on the other hand, gave 10% and 11.6% neutralization at 0 time and 26.3% and 23.3% neutralization at 1 hour, with and without AF serum respectively¹⁸.

Experiment 21. Neutralization of toxoplasma by rabbit antiserum in excess, in the presence of complement, inactivated complement, without complement, and with complement + buffer, by direct assay without dilution.

There are two important changes made in this and the following two experiments, 1) the toxoplasma suspension was diluted so that 0.5 ml of suspension used in both test and control systems contained 5×10^3 toxoplasma, 2) there was no 200-fold dilution carried out. Instead, 0.5 ml amounts of neutralization mixture from the reaction system tubes in the water bath was placed directly onto the monolayers for assay.

17. Contrast for rabbit with AF serum versus rabbit without AF serum is not significant at $\alpha = 0.10$

18. Control versus treatment within rooster with AF serum in significant at $\alpha = 0.10$ p = 0.10

TABLE XIII

NEUTRALIZATION OF TOXOPLASMA BY RABBIT ANTISERUM, WITH AND WITHOUT ACCESSORY FACTOR SERUM

Neutralization time	<u>With accessory factor</u>		Percent neutralization	<u>Without accessory factor</u>		Percent neutralization
	Control Rabbit preinoculation serum, @IFAT-ve +	Rabbit antitoxoplasma serum, IFAT 1:128 *		Control Rabbit preinoculation serum, IFAT-ve	Rabbit antitoxoplasma serum, IFAT 1:128 **	
0 time	82 ‡	82	0	80	83	0
1 hour	90	82	8.89	72	72	0

+ Control system contained 0.5 ml rabbit preinoculation serum instead of rabbit antiserum; otherwise same as test system.

* Test system comprised 0.5 ml suspension containing 1 million toxoplasma in HBSS + 1 ml accessory factor serum + 0.5 ml rabbit antitoxoplasma serum.

** The ratio of toxoplasma suspension : buffer : antiserum = 1:2:1.

‡ Average number of plaques in two replicates.

@ IFAT - indirect fluorescent antibody test.

TABLE XIII Cont'd

NEUTRALIZATION OF TOXOPLASMA BY ROOSTER ANTISERUM, WITH AND WITHOUT ACCESSORY FACTOR SERUM

Neutralization time	<u>With accessory factor</u>			Percent neutralization	<u>Without accessory factor</u>			Percent neutralization
	Control Rooster preinoculation serum, @IFAT-ve	Rooster antitoxoplasma serum, IFAT 1:128 *			Control Rooster preinoculation serum, IFAT-ve +	Rooster antitoxoplasma serum IFAT 1:126 **		
0 time	80†	72		10.0	69	61		11.6
1 hour	76	56		26.32	43	33		23.26

* Test system comprised 0.5 ml suspension containing 1 million toxoplasma in HBSS + 1 ml accessory factor serum + 0.5 ml rooster antitoxoplasma serum.

+ Control system contained 0.5 ml rooster preinoculation serum instead of rooster antiserum; otherwise same as test system.

** The ratio of toxoplasma suspension : buffer : antiserum = 1:2:1.

† Average number of plaques in two replicates.

@ IFA - indirect fluorescent antibody test.

In the test system with complement, besides 0.5 ml of toxoplasma suspension in HBSS-A, 1.0 ml of complement containing 25 C'H50 and 0.5 ml of rabbit antiserum were taken. A small quantity of the same complement was inactivated at 56°C for 30 minutes and was used in 1.0 ml volumes in the tests with inactivated complement and 1.0 ml of buffer (PBS-A with 2% calf serum) in place of complement or inactivated complement, in the tests without complement and in the test system with complement and buffer, an additional 0.5 ml of buffer was used apart from 0.5 ml of toxoplasma suspension, 1.0 ml of complement and 0.5 ml of antiserum.

The respective control systems contained 0.5 ml of rabbit pre-inoculation serum (IFAT -ve) instead of rabbit antiserum.

The results of neutralization at 1 hour's time are given in Table XIV. Maximum neutralization of 81.1% was seen in tubes containing inactivated complement as against nearly equal neutralization observed in tubes, with and without complement, namely 31.4% and 38.1% respectively. When an additional 0.5 ml of buffer was used along with complement, 47.9% as against 31.4% neutralization with complement but without additional buffer was obtained¹⁹.

Experiment 22. Neutralization of toxoplasma by rabbit antiserum in excess, in the presence of complement, inactivated complement and without complement by direct assay without dilution.

But for a few changes, this is a repetition of the preceding experiment. The collecting fluid and the buffer used in this

19. Contrast without complement versus with complement and buffer
= $0.10 < p < 0.20$

TABLE XIV

NEUTRALIZATION OF TOXOPLASMA BY RABBIT ANTISERUM IN EXCESS, IN THE PRESENCE OF COMPLEMENT,
INACTIVATED COMPLEMENT, WITHOUT COMPLEMENT AND WITH COMPLEMENT + BUFFER BY DIRECT ASSAY
WITHOUT DILUTION

Neutralization time	<u>With complement</u>			<u>With inactivated complement</u>			<u>Without complement</u>			<u>With complement + buffer**</u>		
	Control Preinoculation serum +	Antiserum	* Percent neutralization	Control Preinoculation serum +	Antiserum *	Percent neutralization	Control Preinoculation serum +	Antiserum ‡	Percent neutralization	Control Preinoculation serum +	Antiserum*	Percent neutralization
1 hour	51 @	35	31.4	127	24	81.1	126	78	38.1	73	38	47.9

+ Control system contained 0.5 ml rabbit preinoculation serum, (negative in indirect fluorescent antibody test), instead of antiserum; otherwise same as test system.

* Test system comprised 0.5 ml suspension containing 5,000 toxoplasma in HBSS-A + 1 ml complement containing 25 C'H50 and 0.5 ml rabbit antitoxoplasma serum, with a titre of 1:128 in indirect fluorescent antibody test.

‡ The ratio of toxoplasma suspension : buffer : antiserum = 1:2:1.

** Same as the test and control with complement + an additional 0.5 ml PBS-A with 2% calf serum, (buffer), so that the proportion of toxoplasma : buffer : complement : serum = 1:1:2:1.

@ Average number of plaques in two replicates.

experiment was a toxoplasma suspension in HBSS diluted to contain 5×10^3 toxoplasma in 0.5 ml. The test system with complement + buffer was however, omitted and assay for neutralization was carried out besides at 1 hour at 0 time. Complement contained 4 CH'50 in 1 ml.

The results detailed in Table XV reveals certain interesting features. There was neutralization registered at 0 time. In the presence of complement, neutralization was greater both at time 0 and 1 hour, being respectively 20.4% and 53.7% as compared to 11.3% and 24.3% obtained in the absence of complement. It is seemingly at variance with the findings obtained in Experiment 21, where a slightly higher percentage of neutralization (38.1%) was registered by antiserum in the absence of complement than in its presence (31.4%). At the same time, it is also true that 38.1% of neutralization obtained in the absence of complement is considerably lower than the 47.9% obtained in the presence of complement + buffer. It is further delved into under Discussion. As in the preceding experiment, maximum neutralization (61.0%) was once again encountered in the presence of inactivated complement.

Experiment 23. Neutralization of toxoplasma by rooster antiserum to excess, with and without complement by direct assay without dilution.

This is an extension of Experiment 22 with some modifications. Toxoplasma was collected in physiological saline solution (0.85%). The test system with complement comprised 0.5 ml. of suspension containing 5×10^3 toxoplasma in physiological saline solution, 0.5 ml. of PBS-A with 2% calf serum (buffer), 1.0 ml. of

TABLE XV

NEUTRALIZATION OF TOXOPLASMA BY RABBIT ANTISERUM IN EXCESS, IN THE PRESENCE OF COMPLEMENT,
INACTIVATED COMPLEMENT AND WITHOUT COMPLEMENT BY DIRECT ASSAY WITHOUT DILUTION

Neutralization time	<u>With complement</u>			<u>With inactivated complement</u>			<u>Without complement</u>		
	Control Preinoculation serum +	Antiserum *	Percent neutralization	Control Preinoculation serum +	Antiserum *	Percent neutralization	Control Preinoculation serum +	Antiserum **	Percent neutralization
0 time	98 †	78	20.4	164	111	32.3	62	55	11.3
1 hour	54	25	53.7	118	46	61.0	37	28	24.3

+ Control system contained 0.5 ml of rabbit preinoculation serum, (negative in indirect fluorescent antibody test), instead of antiserum; otherwise same as test system.

* Test system comprised 0.5 ml suspension containing 5,000 toxoplasma in HBSS + 1.0 ml complement containing 4 C'H50 and 0.5 ml rabbit antitoxoplasma serum, with a titre of 1:128 in indirect fluorescent antibody test.

** The ratio of toxoplasma suspension : buffer : antiserum = 1:2:1.

† Average number of plaques in two replicates.

complement with 4 CH'50 and 0.5 ml. of rooster antiserum (IFAT 1:64). In the test system without complement, an additional 1.0 ml. of buffer was used in place of complement.

Control systems differed from their respective test systems in having rooster pre-inoculation serum in 0.5 ml. volumes instead of rooster antiserum.

It is clear from the results in Table XVI that rooster antitoxoplasma serum neutralized toxoplasma to a greater degree in the absence of complement both at 0 time and at 1 hour than in the presence of complement. Slightly higher neutralization observed at 0 time than at 1 hour in the presence of complement, namely 33.9% and 32.0% respectively, could only be attributed to experimental error.

TABLE XVI

NEUTRALIZATION OF TOXOPLASMA BY ROOSTER ANTISERUM IN EXCESS, WITH AND WITHOUT
COMPLEMENT BY DIRECT ASSAY WITHOUT "DILUTION"

Neutralization time	With complement			Percent Neutralization	Without complement			Percent Neutralization
	Control Normal serum +		Antiserum *		Control Normal serum		Antiserum ‡	
0 time	112 **		74	33.9	193		98	49.2
1 hour	103		70	32.0	179		76	57.5

+ Control system contained 0.5 ml. normal rooster serum, (negative in indirect fluorescent antibody test) instead of antiserum; otherwise same as test system.

* Test system comprised 0.5 ml toxoplasma suspension containing 5×10^3 toxoplasma in normal saline solution plus 0.5 ml PBS-A with 2% calf serum + 1.0 ml complement containing 4 C'H50 and 0.5 ml rooster anti-toxoplasma serum with a titre of 1:64 in indirect fluorescent antibody test.

‡ The ratio of toxoplasma suspension : buffer : antiserum = 1:3:1.

** Average number of plaques in two replicates.

DISCUSSION

DISCUSSION

It is possible to generalize from the study of growth characteristics of T. gondii in L cells, Vero and secondary CEF monolayers that the rate of growth and the duration of growth of a particular strain of toxoplasma depended on two factors, 1) the cell system used for its growth, and 2) the input multiplicity. In the present study the duration of growth was the shortest in Vero monolayers in which it lasted for 17 days, intermediate in secondary CEF monolayers in which it lasted 21 and 24 days and was the longest in L monolayers in which the infection became persistent. Schuhova (1960) in his report on the long term culture of T. gondii (CB strain) in HeLa cells for over 33 months, observed that during the whole period, the parasites never destroyed all the cells in the culture and that from time to time during the course of growth, the parasites apparently disappeared and the cells showed no signs of degeneration but it was soon followed by typical destruction of cells and the appearance of parasites. This phenomenon was observed when the inoculum size was not too large. A similar feature in HeLa cells in Gey chambers inoculated with smaller number of T. gondii was reported by Lund et al (1963). Whereas total degeneration occurred in 4 to 5 days with heavily infected cultures in their experiments, the cultures infected with lower concentration of parasites were maintained for over 3 months. It seemed to them as if an equilibrium was obtained between the frequency of cell-burst due to parasite multiplication and mitosis of cells in the cultures.

In the present studies, the same phenomenon of chronic state of infection was observed in a new cell-system, namely, L cells at different multiplicities of infection and the features observed were in accord with the findings of Schuhova (1960) and Lund et al. (1963). However, with L cells and T. gondii it looked as if the inoculum size of the parasite need not necessarily be restricted to a small size as evinced from the wide difference in the IM in the first two experiments, ranging from 100:1 in the first to 0.001:1 in the second, and can be explained by the occurrence of multiple infected cells, all the cells of the culture not necessarily becoming infected even with the highest concentration of parasites (Lund et al., 1963).

It is also interesting to note that an equilibrium was reached between the multiplication of parasite and of the cells (L cells) in about 9 days after infection despite infection at different multiplicities in Experiment 3, the total number of extracellular toxoplasma levelling off in about 9 days.

As far as is known, toxoplasma has never been grown in Vero cells. Toxoplasma was grown in Vero monolayers for the first time and it is interesting to find that during the short period of sixteen days of growth, two peaks of growth were observed, the first between 4 and 5 days and the second on day 14 after infection. The increase in the number of toxoplasma was over one log unit. (Vide Figure 4).

There has been no report on Vero cell cultures being used to propagate toxoplasma in vitro. Macfarlane and Sommerville (1969) found that Vero cells, derived from kidney epithelium of Cercopethicus aethiops (an African green monkey) and designated by Yasamura and

Kawakita (1963), were able to grow a wider range of viruses than any other cell-line known, with development of cytopathic effect in almost every instance and they recommended that Vero was superior in overall performance to primary human amnion cells, HEp-2, HeLa, and primary monkey kidney cells. Further, Vero is said to enjoy the advantage of complete freedom from contaminating simian viruses and consistency of performance unlike primary monkey kidney cells. The present growth experiments and the plaque assay of toxoplasma in Vero cell cultures have yielded results in confirmity with the findings of Macfarlane and Sommerville (1969) and have therefore been used in the in vitro neutralization of toxoplasma by plaque assay experiments with advantage.

Working on primary CEF monolayers, Chaparas and Schlesinger (1959) obtained a higher efficiency of plating as they decreased the size of inoculum, getting a maximum increase of over 3 log units in 96 hours. Their study did not extend beyond 96 hours of growth. In the present study, in the two experiments with secondary CEF, the duration of growth lasted 21 days when the IM was 25:1, whereas it remained for 24 days when the IM was lower, namely 10:1 and the peak in the former was reached on post-inoculation day 14 with an increase of 1 log unit in the total number of live, extracellular toxoplasma (Vide Figure 5).

The efficiency of preservation of toxoplasma in liquid nitrogen as judged by the results obtained in the preliminary Experiment number 6 with plaque assay of: 1) toxoplasma derived from the tissue culture fluid and preserved in 10% DMSO and 7.5% calf serum and 2) toxoplasma from the peritoneal exudate of mice infected 72

hours earlier, preserved in 20% DMSO and 20% calf serum for 137 and 135 days respectively, was rather disappointing in that a survival of 1 PFU for every 3×10^6 toxoplasma initially preserved for about $4\frac{1}{2}$ months was obtained in both the samples. Plaquing efficiency in respect to toxoplasma from mice infected 72 hours earlier was about 1% (Vide Experiment 8). On this basis, it may be said that about 100 toxoplasma survived out of every 3×10^6 toxoplasma originally preserved or about 30 survivors for every 1×10^6 toxoplasma. PE of 1% might reasonably be true for toxoplasma from tissue culture fluid also, considering that Lund et al (1963) recovered only 1% of counted parasites as TCID₅₀ in their experiment in HeLa cells using T. gondii (RH).

Experiment 7, in which toxoplasma from mice infected 48 hours earlier instead of 72 hours earlier, was preserved in various freeze mixtures, yielded more accurate results by virtue of the fact that a known number of PFU of toxoplasma was preserved and the survivors were also estimated in terms of PFU. The highest survival rate of 0.42% was observed in 5% DMSO and 10% calf serum. Even though this may appear not very encouraging at first sight, it ranks as the best result reported in the study of preservation of toxoplasma in view of the fact that out of 1.1×10^6 PFU (about 3×10^6 toxoplasma) originally preserved, the survivors amounted to 4,600 PFU. Notwithstanding all this, it must be confessed that it is hard to compare these figures with any data from other workers, for a perusal of literature on preservation of toxoplasma would reveal no clear cut data.

Chandler and Weinman (1956) reported an attrition of "well over 99%" when they preserved T. gondii (RH) and a porcine strain obtained from the peritoneal exudate of mice in 15% glycerol at -70°C. for 45 to 185 days. They demonstrated the presence of toxoplasma among survivors by mouse inoculation and concluded that starting with a sufficient number of organisms (1.7×10^7) recovery of organisms in mice was accomplished after storage for 122 days. Paine and Meyer (1969) took T. gondii grown in bovine kidney cell cultures (MDBK) for preservation at liquid nitrogen temperature in 7% DMSO + 25% foetal calf serum and were able to conclude that the presence of viable, infectious toxoplasma was demonstrated by mouse inoculation and confirmed by microscopic examination of fresh ascitic fluid and of stained smears after storage for 6, 87 and 333 days.

Foley and Remington (1969) reported obtaining higher plaquing efficiency and reproducible results using artificially lysed toxoplasma obtained from mice infected 48 hours earlier, whereas toxoplasma from mice infected 72 hours earlier was reported to have given low plaquing efficiency and the results were not reproducible either. Chaparas and Schlesinger (1959) claimed greater efficiency when they used toxoplasma by artificial lysis of infected CEF cells. While greater PE for artificially released toxoplasma is explained by the fact that toxoplasma deteriorate the longer they remain extracellularly, detailed comparative data are not forthcoming. Lycke and Lund (1964b) observed that toxoplasma released from host cells would commence to disintegrate unless they penetrated another host cell without delay. The results obtained in the present study

revealed a higher PE for "48 hours" toxoplasma than for "72 hours" toxoplasma. However, reproducibility of PE for "48 hours" toxoplasma was rather difficult (Vide Table V). A higher PE was obtained when the "48 hours" toxoplasma was artificially lysed than when not lysed. It is interesting, however, to note that the converse was true when the "72 hours" toxoplasma was artificially lysed, the PE obtained being lower when artificially lysed than when not lysed. It is rather disturbing and difficult to explain unless one takes cognizance of the fact that antitoxoplasma antibodies could make their appearance in the peritoneal exudate of infected mice in about 72 hours' time, but not in 48 hours (Frenkel, 1956). In the former case it looks as if, in the presence of antibodies, the intracellular toxoplasma when artificially released (majority of whose population may not be fully mature) probably deteriorate far more than the mature adult toxoplasma found extracellularly after the natural process of cell-bursts.

Studies of in vitro neutralization by plaque assay have revealed many interesting features for discussion. There was no neutralization of toxoplasma whatever by rabbit and rat antisera at 5 minutes, 20 minutes and 1 hour of neutralization in the absence of complement (Vide Experiments 16 and 17, Tables VII and VIII). In Experiment 16, the rabbit antitoxoplasma serum had a low IFAT titre of 1:32 but in later experiments, when higher titred antiserum (IFAT 1:128) was used, the same results were obtained when the standard method of neutralization with dilution was employed. Rooster antiserum however, differed in this respect from both rabbit and rat antisera in that there was significant neutralization in the absence.

of guinea pig complement at 1 hour. The most unusual yet interesting feature of rooster antiserum is its ability to produce higher neutralization without complement than with complement and it is particularly significant in Experiment 19 (Table XII) where it was nearly twice as much as that obtained with complement. (Contrast with complement versus without complement - significant at $\alpha = 0.10$ $0.05 < p < 0.10$)

Lycke and coworkers (1965) using human antitoxoplasma serum, carried out a series of tests to find out the effect of immune serum and various components of complement and of accessory factors on the infectivity of toxoplasma in cell cultures, taking the relative number of infectious units (RNIU) as seen under the phase contrast microscope as the criterion, and reported that heated immune serum, and heated human immune serum + activator serum mixture, revealed considerable neutralizing capacity which was contrary to the neutralization observed in the in vitro dye test in which the complement factors C'1 C'2 C'3 and C'4 (Feldman, 1960) and Mg⁺⁺ ions and pro-perdin (Feldman, 1956) were found to be necessary for the antibody effect. In other words, considerable neutralizing capacity was evident with human antiserum in the absence of complement and in the absence of thermolabile factors of complement in the tissue culture unlike in the tube DT. In the present study of neutralization by plaque assay in Vero monolayers, there was no neutralization demonstrable in either rabbit or rat antisera in the absence of complement. However, rooster antiserum showed considerable neutralizing capacity in the presence of complement and surprisingly greater neutralizing capacity was elicited in the absence of comple-

ment. Complement supplied in the form of guinea pig serum is known not to activate rooster antiserum unlike rabbit or rat antiserum (Brumfield et al 1961, Benedict, 1967). The comparatively less neutralization observed in the presence of complement than in its absence is therefore believed to be due to the effect of inhibitors of complement supplied along with the complement in the guinea pig serum (Lepow et al 1965). However, when rabbit antiserum was used proportionately in excess of toxoplasma concentration and the neutralizing mixture was assayed directly without 1-200 dilution, there was neutralization manifested both with and without complement in 1 hour's time. In one such experiment (Experiment 22, Table XV), rabbit antiserum exhibited greater neutralization in the presence of complement than in its absence and apparently the opposite was true in Experiment 21 (Table XIV). This disparity in the results of these two experiments is difficult to explain since the conditions in both the experiments were not identical, particularly the absence of Ca^{++} ions in HBSS-A used as collecting fluid in Experiment 21 and its presence in HBSS used as collecting fluid in Experiment 22. Also complement in 1.0 ml. contained 25 CH'50 in the former and 4 CH'50 in the latter. Lastly, it must be stressed that the buffer used in Experiment 21 contained 2% calf serum and the buffer was missing in the test system with complement while it was used in place of complement in the test system without complement. So it becomes incumbent in Experiment 21 to compare the results of neutralization of tests without complement (but with buffer) with that of test with complement + buffer (see Table XIV), when it becomes clear that the rabbit antiserum is capable of producing greater neutra-

lization in the presence of complement than without complement. In other words, rabbit antiserum, unlike rooster antiserum, is able to fix guinea pig complement in the presence of toxoplasma. Rooster antiserum, on the other hand, exhibited greater neutralizing capacity in the absence of complement both in the standard method with dilution and in the direct method without dilution (see Tables VIII, XI, XIV and XVI).

Rabbit antiserum produced 44.2% neutralization in the presence of complement by the standard method in experiment 17 (Table VIII) and 47.9% by the direct method without dilution in experiment 21 (Table XIV). In these two experiments the titre of the antiserum, the diluent and the amount of complement used were one and the same. It is important to note that the excess of antiserum used in the direct method made no significant difference in neutralization to that obtained by the standard method in the presence of complement. This shows that neutralization by the standard method was also carried out in antibody excess though the relative amount of antiserum to the number of toxoplasma in the reaction mixture was 200 times less than in the direct method. In addition, neutralization appeared to be completed within one hour. Absence of 1-200 dilution in the direct method should permit the antiserum to continue to effect neutralization even after one hour allowed for neutralization in the reaction tube to produce higher neutralization than in the corresponding standard method if complete neutralization did not take place within one hour but it was not the case. In a similar set of experiments, namely, 18 and 22 (Tables X and XV) with HBSS as diluent, the results obtained with rabbit antiserum in the presence of complement containing 4 C'H-50 are at variance. However, comparison of results of experiments 19 and 23 (Tables XI and XVI), in both of which rooster antiserum was employed under identical conditions in the two methods of neutralization will bear out the

above inferences. Further, dilution of 1-200 was, probably, instrumental in bringing about dissociation of the antigen-antibody complex in the absence of complement in the case of rabbit antiserum. Yet, since more than one variable is involved, these findings need confirmation. Lastly, that neutralization of toxoplasma by rabbit antiserum is augmented in the presence of guinea pig complement may be accepted as established.

Strannegard (1967b) studied immunoinactivation of toxoplasma under an electron microscope with the aid of ferretin-labelled antibody of 7S and 19S fractions of rabbit antitoxoplasma serum and reported that in the absence of AF serum, the antibodies were distributed on the surface of toxoplasma whereas in the presence of AF serum, the antibodies were observed in the interior of parasites consequent on extensive damage of the parasite membrane. Accordingly, it is reasonable to expect neutralization in the presence of AF serum but not necessarily in its absence. With the antisera of IFAT titre of 1:128 used in the study, it seems to be true for rabbit antiserum but not for rooster antiserum (Table XIII). However, contrast for rabbit with AF serum versus rabbit without AF serum is not significant at $\alpha = 0.10$; $0.30 < p < 0.40$.

Another yet striking feature in the neutralization study at hand is the maximum neutralizing capacity evidenced with the rabbit antitoxoplasma serum in the presence of inactivated guinea pig complement both in the standard neutralization by dilution method and in the antibody excess combined with direct assay without dilution method (Tables XIV and XV). Lycke et al., (1965) obtained the most effective neutralization in their tissue culture study when they employed mixtures of immune serum and AF serum. They encountered reduction in the neutralizing capacity when C'1 and C'2 were destroyed by heat, and considerable reduction when C'4 was removed by treatment

with NH_4OH as well as when Mg^{++} ions were removed by EDTA from the human antitoxoplasma serum-AF serum mixture. Furthermore, the largest reduction in neutralizing capacity followed when C'3 was absorbed with zymosan. Guinea pig complement was inactivated at 56°C for 30 minutes in the present study and would be devoid of C'1 and C'2⁽²¹⁾, the thermolabile factors of complement. The loss of the thermolabile factors was expected to result, on the above basis, in a decrease in the neutralizing capacity of the anti-serum. However, the results of the experiments (Tables X, XIV and XV) indicate otherwise. There are several inhibitors of complement, such as inhibitors of C'1, C'3, C'3a, C'4, C'5a and C'6 found in the sera of various species of animals (Muller-Eberhard, 1969). Inhibitors of C'4 and C'6 are found in normal guinea pig serum. While C'4 and C'6 are heat-stable, the inhibitors of C'4 and C'6 are heat-labile (Opferkuch *et al.*, 1968; Tamura and Nelson, 1967). Inactivated guinea pig serum would, therefore, contain C'4 and C'6 free from the inhibitory influence of their respective inhibitors to exercise neutralization effect on toxoplasma and this, probably, accounted for the enhanced neutralization observed in the presence of inactivated guinea pig complement.

Neutralization of toxoplasma by rabbit- and rooster-antisera with and without AF serum (Table XIII) again revealed that rabbit antiserum behaved differently from rooster antiserum, not unlike the results in experiment with and without complement. Here again, the rabbit antiserum showed no neutralization in the absence of AF serum, whereas the presence

21. In 1965 when Lycke *et al.*, reported the results of their study, guinea pig complement was known to comprise of only 4 components, namely, C'1, C'2, C'3 and C'4, of which C'1 and C'2 were thermolabile. As of now, complement consists of 9 components, out of which C'1, C'2 and C'5 are known to be heat-labile and C'8 and C'9 are relatively heat-labile (Polley and Muller-Eberhard, 1966). This disparity, however, does not affect the discussion.

or absence of AF serum made little difference with rooster antiserum, neutralization being manifested in either case in about equal degree. The slight neutralization of 10% and 11.6% seen at 0 time under rooster antiserum with and without AF serum respectively in Table XIII could be due to the relative delay in assaying immediately after mixing the parasite suspension and the antiserum-AF serum mixture. Nevertheless, it points out the speed with which rooster antiserum commences neutralization.

Higher percentage of neutralization obtained by rooster antiserum compared to that obtained by rabbit antiserum, both of which had the same IFAT titre of 1:128 in experiment 20 (Table XIII), is suggestive not only of the difference of species from which antisera were obtained but also that IFAT was not detecting either all the components of neutralizing antibody or not at all. Further, the fact that rooster antiserum is likely to consist of predominantly 19S antibodies than 7S antibodies (Uhr et al., 1962; Benedict, 1967) and the observations of Strannegard (1967a) that 19S antibody type was more effective per molecule than 7S antibody in the immunoinactivation of toxoplasma, lend support to the present findings of higher neutralization with rooster antiserum than with rabbit antiserum.

Considerable neutralization of toxoplasma at 0 time by both rabbit and rooster antisera in excess, on direct assay without dilution (Tables XV and XVI) was in all probability due to the fact that antiserum in excess had a period of three hours allowed for adsorption of toxoplasma prior to overlay, within which to neutralize toxoplasma since there was no 1-200 dilution in this method to overcome the antibody effect at 0 time. In this method without dilution, the neutralization time, therefore, becomes superfluous.

SUMMARY

SUMMARY

Study of growth characteristics of T. gondii in L, Vero and secondary CEF monolayers revealed that the rate and duration of growth of toxoplasma depended on: 1) the cell system used for its growth, and 2) the IM of toxoplasma. Growth period was the shortest in Vero, intermediate in secondary CEF and was apparently persistent in L cells despite the wide range of IM of toxoplasma in the latter.

Plaque assay in Vero monolayers, for the first time, yielded a highly satisfactory assay system for toxoplasma, being found better than L, secondary CEF, and BHK cells that were tested.

Efficiency of preservation of toxoplasma at liquid nitrogen temperature was evaluated using toxoplasma from tissue culture fluid, from peritoneal exudate of mice infected 72 hours earlier and 48 hours earlier, in various freeze mixtures. Best results were obtained when toxoplasma obtained from mice infected 48 hours earlier was preserved in liquid nitrogen in a freeze mixture containing 5% DMSO and 10% calf serum as judged by the plaque formers.

Both the use of chicken antitoxoplasma serum employed in the in vitro neutralization by plaque assay and the in vitro neutralization by plaque assay of toxoplasma are new ideas. Standard method of neutralization with dilution and neutralization in antibody excess and without dilution were performed using rabbit, rat and rooster antisera in the former and rabbit and rooster antisera in the latter. Rabbit and rat antisera with an IFAT titre of 1:128 required guinea pig complement to produce any neutralization by the standard method unlike

neutralization in antibody excess and without dilution method in which rabbit antiserum did produce neutralization in the absence of complement and enhanced neutralization in its presence. Rooster antiserum, on the other hand, showed greater capacity for neutralization in the absence of complement than when complement was present in both the methods of neutralization. It is interesting to find that rabbit antiserum exhibited maximum neutralizing capacity when inactivated guinea pig complement was added and assayed by both the standard and direct methods.

When accessory factor serum was used in place of guinea pig complement, the pattern of neutralization by rabbit as well as rooster antiserum, with and without AF serum was not unlike that seen with and without complement, the degree of neutralization, however being of a lower degree when AF was used.

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APPENDICES

APPENDIX A

RIF Growth Medium @

Sterile distilled water	675.7 ml.
Medium 199 (10X)*	82.0 ml.
Sodium bicarbonate (2.8%)	29.5 ml.
MEM amino acids (50X)*	16.4 ml.
MEM vitamins (100X)*	8.2 ml.
MEM glutamine (100X)*	8.2 ml.
Tryptose phosphate broth ⁺	100.0 ml.
Calf serum (inactivated at 56°C. for 30 minutes)	80.0 ml.
Adjust pH to 7.3 with 1N Na(OH)	
Take sterility	
Add Penicillin 200 units/ml., Streptomycin 200 μ g/ml., Amphotericin B. 2.5 μ g/ml. (PSF), (100X)	10.1 ml.
Store in freezer	

@ Dr. A. M. Wallbank, personal communication

* Grand Island Biological Company

+ Difco Laboratories

APPENDIX B

BPRIF Medium

BPRIF is growth medium (RIF) in which calf serum is replaced by 5% Bacto-peptone. (Difco Laboratories)

APPENDIX C

Maintenance Medium (MM)

Maintenance medium consists of 1 part of growth medium and 9 parts of Medium 199 (IX).

APPENDIX D

Agar Overlay

Earle's balanced salt solution (5X)	45.3 ml.
Distilled water	25.0 ml.
MEM amino acids (50X)	11.0 ml.
MEM vitamins (100X)	5.5 ml.
MEM glutamine (100X)	5.5 ml.
MEM non-essential amino acids (100X)	5.5 ml.
Calf serum (inactivated at 56°C for 30 minutes)	22.0 ml.
Tryptose phosphate broth	27.5 ml.
Sodium bicarbonate (2.8%)	18.5 ml.
PSF (100X) (See Appendix A)	2.75 ml.
Bacto-agar (1.8%)	110.0 ml.

Note: The first ten components were mixed in a sterile flask and heated to 45°C in a water bath and mixed with Bacto-agar which was cooled to 45°C.

APPENDIX E

Neutral Red Agar (NRA)*

PBS-A (10X) (See Appendix F)	100.0 ml.
Add about 750.0 ml. distilled water to help dissolve Neutral red dye	0.35 gm.
Distilled water, q.s.	1000.0 ml.
Mix for $\frac{1}{2}$ hour on magnet stirrer	
Filter through filter paper into 1,500 ml. flask	
Difco agar	8.0 gm.
Bring to boil on heater-magnetic stir plate	
Autoclave at 121°C for 15 minutes	

* Dr. A. M. Wallbank, personal communication

APPENDIX F

Dulbecco's Phosphate Buffer Solution-A (PBS) 10X

Sodium chloride	80.0 gm.
Potassium chloride	2.0 gm.
Na ₂ HPO ₄ (anhydrous)	11.5 gm.
KH ₂ PO ₄	2.0 gm.
Water q.s. to	1,000.0 ml.
Autoclave at 121°C for 30 minutes	

APPENDIX G

Dulbecco's Phosphate Buffer Solution (PBS)

Solution-A (10X) (See Appendix F)

Solution-B (100X)

Ca Cl₂ 5.0 gm.

Water, q.s. to 500.0 ml.

Solution-C (100X)

Mg Cl₂ 6 H₂O 5.0 gm.

Water, q.s. to 500.0 ml.

Place 100.0 ml. of Solution-A (10X) in a 1 liter volumetric flask about three-quarters full of distilled, deionized water. Slowly add with stirring 10.0 ml. of Solution-B and 10.0 ml. of Solution-C. Finally, q.s. to 1 liter with distilled, deionized water.

To sterilize, filter it through a 0.22 μ m Membrane filter. Check the sterility and hold for one week.

APPENDIX H

Hanks' Balanced Salt Solution-A (HBSS-A) (10X)

NaCl	80.0 gm.
KCl	4.0 gm.
MgSO ₄ ·7H ₂ O	2.0 gm.
Na ₂ HPO ₄	0.5 gm.
Glucose	10.0 gm.
KH ₂ PO ₄	0.6 gm.

Q.s. to one liter with distilled water

APPENDIX I

Hanks' Balanced Salt Solution (HBSS)

Solution-A (10X) (See Appendix H)

Solution-B (10X)

CaCl₂ 1.4 gm.

q.s. to one liter with distilled, deionized water

Solution-C, 2.8%

NaHCO₃ 14.0 gm.

q.s. to 500.0 ml. with distilled water

For one liter of HBSS, add 100.0 ml. of Solution-A to 700.0 ml. of distilled, deionized water. To the preceding, add 100.0 ml. of Solution-B slowly with mixing and 4.0 ml. of phenol red. Make up to one liter in a volumetric flask with distilled, deionized water.

Place the contents of the volumetric flask in a two liter flask for autoclaving, plug flask with cotton and cover with aluminum foil top. Autoclave at 121°C for 30 minutes. When solution is cool add 12.5 ml. of 2.8% sodium bicarbonate.

APPENDIX J

Alkaline Methylene Blue Dye Solution (Sabin and Feldman, 1948)

Count of toxoplasma in the mouse exudate suspension as well as in the tissue culture fluid was carried out at 1+9 dilution with freshly prepared alkaline methylene blue dye solution which was prepared as follows:

1. Stock solution of methylene blue

Prepare a saturated solution of methylene blue in 95% ethyl alcohol

2. Alkaline buffer solution for methylene blue

Na_2CO_3 (0.53% aqueous solution) 9.73 ml.

$\text{Na}_2\text{B}_4\text{O}_7$ (1.91% aqueous solution) 0.27 ml.

The Na_2CO_3 and $\text{Na}_2\text{B}_4\text{O}_7$ stock solutions should be kept separately and mixed fresh on the day of use.

Mix 1 part of stock solution of methylene blue with 10 parts of alkaline buffer solution.

APPENDIX K

Accessory Factor

This is normal human serum shown to contain "accessory factor", a heat-labile substance which is necessary for the action of the antibody on toxoplasma organisms in the dye test. The presence of accessory factor is demonstrated by using the serum as diluent for the organisms and mixing with known positive antitoxoplasma serum which has been inactivated. If the inactivated serum recovers its ability to prevent staining of the organisms in the test, accessory factor is present. Not all human serum is effective. Accessory factor serum keeps well if frozen at -70° but quickly deteriorates on exposure to heat.