THE UNIVERSITY OF MANITOBA

CHARACTERIZATION OF FIVE STRAINS OF TOXOPLASMA GONDII BY RECIPROCAL NEUTRALIZATION IN CELL CULTURE, VIRULENCE IN MICE, IMMUNODIFFUSION AND IMMUNOELECTROPHORESIS.

BY

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P.T. SHETTIGARA

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

The innumerable strains of toxoplasma isolated from humans, animals, and birds appeared to belong to a single species of Toxoplasma gondii (T. gondii) despite several previous attempts at classification by others.

The objective of the study was to test evidence of strain specificity among different strains of T. gondii. Accordingly, five strains of toxoplasma were examined for strain specificity employing three methods, namely, reciprocal in vitro neutralization in Vero monolayers, virulence of individual strains in mice as judged by median lethal dose (LD50) and median response time (RT50) values, and a comparative study of strain-specific precipitating toxoplasma antibodies in immunodiffusion and immunoelectrophoresis.

Neutralization of toxoplasma by rabbit antiserum was found to reach maximum proportions in the presence of heat inactivated guinea pig complement. Addition of anitgamma globulin gave disappointing results. Optimum pH of Hanks' balanced salt solution was found to be 7.7, and the optimum amount of complement 8 C'H50 units. Early antiserum gave much lower percentage of neutralization than hyperimmune antiserum. Reproducibility was rather poor in dilution method on account of dissociation of toxoplasma from its antibody on dilution which was confirmed in experiments with varying concentration of toxoplasma. It was demonstrated that there was no soluble factor(s) to which dissociation could be attributed in toxoplasma suspension.

Reciprocal neutralization of five strains of toxoplasma with strain specific antisera in the presence of inactivated complement was carried

out and from the normalized neutralization values obtained the five strains were classified into 4 groups, C37 and Beverley strains forming one group and RH, V16 and C56 strains forming three separate groups on analysis with Chi-square test.

Study of the relative virulence of the strains as judged by the normalized virulence values derived from LD50, RT50, and the product of LD50 and RT50 enabled distinction of each strain from the other except V16 from C56, and C37 from Beverley.

Using two types of antigenic preparations, namely, supernatants and tissue culture toxoplasma antigens in immunodiffusion it was not possible to differentiate among the strains. Evidence for non-specific absorption of toxoplasma antibodies by lyophilized normal mouse serum was apparent. Presence of precipitating toxoplasma antibodies in the antisera was convincingly shown employing tissue culture toxoplasma antigens in immuno-diffusion and the presence of specific antibodies in antiRH serum in indirect micro-radioimmunoassay.

Toxoplasma supernatant antigens gave additional bands as compared to normal mouse peritoneal exudate on diffusion with strain specific antisera after electrophoresis. Study of immunoelectrophoretic pattern of each antigen-antiserum combination appeared to provide a method for distinguishing the strains of toxoplasma.

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List of Abbreviations

ACGG	Anti chicken gamma globulin
ADD	Average day of death
AF	Accessory factor
AMB	Alkaline methylene blue
ARGG	Anti rabbit gamma globulin
CAM	Chorioallantoic membrane
CEF	Chick embryo fibroblast
CF	Complement fixing or complement fixation
CFT	Complement fixation test
DMSO	Dimethyl sulfoxide
DT	Dye test
DW	Distilled water
ED50	Median or fifty per cent effective dose
EDTA	Ethylenediaminetetraacetic acid
FCA	Freund's complete adjuvant
GP	Guinea pig
HBSS	Hanks' balanced salt solution
HSV	Herpes simplex virus
IEP	Immunoelectrophoresis
IFAT	Indirect fluorescent antibody test
LD50	Median or fifty per cent lethal dose
MEM	Minimum essential medium
MPA	Micro precipitation in agar
NME	Normal mouse (peritoneal) exudate
NMS	Normal mouse serum
NRPIS	Normal rabbit preimmune serum
PBS	Phosphate buffer solution
PFU	Plaque forming units
PSF	Penicillin, streptomycin, and fungizone
RIA	Radioimmunoassay
RT50	Median or fifty per cent response time
TC	Tissue culture (cell culture)
T. gondii	*Toxoplasma gondii*

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CHARACTERIZATION OF FIVE STRAINS OF TOXOPLASMA GONDII BY RECIPROCAL NEUTRALIZATION IN CELL CULTURE, VIRULENCE IN MICE, IMMUNODIFFUSION AND IMMUNOELECTROPHORESIS.

INTRODUCTION AND STATEMENT

OF

THE PROBLEM

Toxoplasmosis is a zoonotic disease caused by a protozoan parasite, Toxoplasma gondii, (T. gondii) which affects almost all mammals and birds throughout the world and is characterized by protean symptoms in either congenital or acquired forms.

T. gondii occurs in three forms: tachyzoite, cyst, and oocyst. The oocyst is a faecal form found in the faeces of domestic cats (and other felidae), and since it is quite resistant to environmental hazards, unlike the other two forms, it plays an important role in the transmission of the disease through faecal contamination. (Work, 1971; Wallace, 1971, 1972 a, 1972 b and 1973). Carnivorism and transplacental transmission are additional modes of the spread of toxoplasmosis.

The infection is widespread throughout the world, its prevalence being higher in warmer and humid areas and at lower altitudes than in arid and colder areas and at higher altitudes (Feldman et al., 1956 a; Walton et al., 1966). The frequency of infection is seen to increase with advancing age in humans as well as in animals. It has been observed with equal frequency among vegetarians and meat-eaters (Rawal, 1959).

Acquired clinical toxoplasmosis is rarely encountered in humans. Human congenital toxoplasmosis is a serious disease resulting in abortion, premature birth, still-birth, or neonatal death. An incidence of 1 in 500 deliveries is reported in the USA (Hume, 1972) and in Paris, it is about 10 per 1,000 per year (Desmonts and Couvreur, 1974).

For infection to be transmitted to the foetus, the mother must acquire the infection during pregnancy. Direct and indirect contact with the domestic cat, the definitive host for T. gondii is the pivotal factor in this. Preventive measures include consumption of well-cooked

meat, proper handling of raw meat using rubber gloves, keeping cats indoors to prevent them from hunting, feeding cats with dry, canned or cooked meat, and daily disposal of cat-litter.

The consensus of opinion has it that there is only one species and only one serotype of toxoplasma (Feldman, 1968; Beattie, 1967; Robertson, 1962; and Jacobs, 1967). While innumerable strains of toxoplasma have been isolated from humans, animals and birds, all strains of toxoplasma 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 (dye test) using human and hare strains as antigen (Christiansen & Siim, 1951), in vitro neutralization tests (dye tests) employing antisera produced in rats against various strains (Wildfuhr & Hudemann, 1952), in vivo neutralization tests by challenging passively immunized mice with graded doses of toxoplasma, in vitro neutralization of toxoplasma followed by innoculation of groups of mice for death rate and survival times (Nikkels, 1965) and polyacrilamide-gel electrophoresis (Bloomfield & Remington, 1970) employed to distinguish different strains into immunologic- or sero- types have failed to distinguish the strains.

In an attempt to determine a precise and sensitive cell system for plaque assay of toxoplasma, Vero cell monolayer was found satisfactory and superior to mouse L fibroblasts, secondary chick embryo fibroblast and baby hamster kidney cell monolayers (Shettigara, 1971). Further, basic aspects of in vitro neutralization of toxoplasma by plaque assay was studied using antitoxoplasma serum prepared in rabbits, rats and roosters in the absence and in the presence of guinea pig (g p) serum as complement as well as in the presence of inactivated g p complement. Both the stan-

dard method of neutralization with "dilution" and the direct method without dilution were performed. Rabbit antiserum showed greater neutralizing capacity in the presence of heat inactivated g p complement than in the presence of uninactivated g p complement or in its absence (Shettigara, 1971).

The objective of the present study was to investigate the possibilities of characterizing five different strains of T. gondii in order to find out whether there is any evidence of strain specificity among them. For this purpose, three different approaches were made; firstly, reciprocal in vitro neutralization was undertaken by plaque assay in Vero monolayers employing hyperimmune sera prepared in rabbits against each of the five strains. Quantitative study of each strain specific antiserum in its ability to neutralize the homologous and heterologous strains was examined after 8 hours' neutralization in test tubes at 37°C, the extent of neutralization being the proportion of survivors of toxoplasma which are plaque-formers in the test system as compared with the control system in which normal preimmune rabbit serum was used in place of rabbit antitoxoplasma serum. Secondly, the property of virulence of each strain was studied. To overcome the inaccuracy inherent in the count of extracellular, live toxoplasma in the haemocytometer under the light microscope, the 50 per cent lethal dose (LD50) of each strain for mice was determined in terms of plaque forming units (PFU) in Vero monolayers. Similarly the 50 per cent response time (RT50) in respect of each strain of toxoplasma was studied in Swiss White mice. From the above data, virulence of each strain was deduced as a product of reciprocals of LD50 and RT50, and the relative virulence of the five strains studied was compared for distinction from one another after normalizing the virulence values obtained.

Thirdly, immunodiffusion by the method of Ouchterlony (1949) and immunoelectrophoresis (IEP) were employed in the study using different preparations of strain specific toxoplasma antigen and rabbit antisera to explore the feasibility of this method as a tool to distinguish the differences in the antigenic determinants of individual strains of T. gondii.

Practical applications of the study are many. Recognition of specificity of a strain of toxoplasma through either virulence or neutralizing ability or immunodiffusion and IEP or a combination of these, would be of immense aid in epidemiological investigations directed to tracing back and forth of the source and extent of spread of infection in order to facilitate execution of necessary preventive measures in the event of an outbreak of toxoplasmosis. It can be employed with advantage in studies on transmission of toxoplasma and, in etiological studies by being able to relate the specific strain to certain specific clinical entity.

Another practical application is to investigate the possible role of strain specific antiserum in the therapy of animals or humans suffering from acute congenital or acute acquired toxoplasmosis either alone or in combination with drugs.

Finally as a test for toxoplasma antibody, neutralization of infectivity as determined in cell cultures has the twin advantages of fairly high sensitivity and direct measurement of antibodies of significance in immunity.

LITERATURE REVIEW

I. THE PARASITE

T. gondii was first recognized in *Ctenodactylus gondi* by Nicolle and Manceaux in 1908. It occurs in three forms: 1) Tachyzoite or proliferative form; 2) Cyst form, and 3) Oocyst.

1) The tachyzoite or proliferative form is motile. It is oval or crescent in shape and measures 2-4 x 4-7 μ in size. The anterior end is pointed and the posterior rounded end contains the nucleus. It is found most frequently in reticuloendothelial cells. It is an obligate, intracellular parasite which can invade and multiply in nucleated cells of all tissues of all mammals and birds. *T. gondii* as seen within the mouse erythrocytes which are not nucleated has been, however, reported by Jadin *et al*. (1967). Recently, Ogunba (1972) has observed toxoplasma in the nuclei of cells in tissue culture.

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 organism (Goldman *et al*., 1958).

2) The cyst is 30-100 μ in diameter. It contains as many as 3,000 individual bradyzoites or cystizoites and is bounded by a double layered cyst wall. Cysts are found chiefly in the brain and lungs and are spherical whereas in the cardiac and skeletal muscles they are elongated. Cysts arise from proliferative forms in later stages of acute infection, during sub-acute and possibly during chronic infections. They can persist for years in the tissues and, when intact are usually unaccompanied by cellular reaction.

3) The oocyst is a faecal form of the parasite which was detected recently in the cat faeces. It has remarkable resemblance to coccidian oocyst of Isospora (Work and Hutchison, 1969; Frenkel et al., 1969; Sheffield and Melton, 1969). It measures 9 x 14μ, is ovoid. Unlike the cyst and the tachyzoite, oocysts tolerate wide limits of osmotic pressure changes and remain viable at room temperature for at least twelve months (Work, 1971).

II. ACQUIRED TOXOPLASMOSIS

While the vast majority of acquired toxoplasmosis in man are symptomless and are recognized by finding serum antibodies (Beverley, 1969), there have been overt cases reported from time to time. Acquired toxoplasmosis may present in three main forms - simple lymphadenopathy, lymphadenopathy with involvement of another organ, or generalized toxoplasmosis. Generalized toxoplasmosis may be encountered in accidental laboratory infections or in patients undergoing immunosuppressive therapy or as secondary to such conditions as Hodgkin's disease, leukemia or lymphosarcoma in which immunological deficiency is met with (Beverley, 1973).

III. CONGENITAL TOXOPLASMOSIS

Since the first description of congenital toxoplasmosis in a child by Wolf et al., (1939), many cases have been reported from different parts of the world. The so-called classical tetrad of neonatal toxoplasmosis are chorioretinitis, hydrocephaly or microcephaly, intracranial calcification and psychomotor retardation (Sabin, 1941). However, this tetrad has since been also found compatible with cytomegalic inclusion disease.

Couvreur and Desmonts (1962) have shown that congenital toxoplasmosis can be often inapparent at birth.

There are two schools of thought in regard to the pathogenesis of human neonatal toxoplasmosis. Workers in Eastern Europe and West Germany believe that T. gondii is one of the main infectious causes of repeated abortions in women. On the other hand, in the experience of Eichenwald et al., (1954), Feldman et al., (1956 b), and Desmonts et al., (1965), there is no record of a woman giving birth to more than one toxoplasmic child. According to them, it is only when a woman acquired her primary infection during pregnancy that she could pass it on to her foetus, and even then, only about 50% of the children are infected and that subsequent pregnancies are not affected.

IV. EPIDEMIOLOGY OF TOXOPLASMOSIS

The important characteristics of the biology of toxoplasma lie in the universal distribution, its capacity for infecting animals of different zoological classes and its ability for parasitism in practically all the animal tissues. It is probable that infection is frequent but disease is exceptional in man and animals (Beattie, 1960). Surveys using skin test and serological tests show an increasing prevalence with advancing age in humans and animals, which suggests that continuing exposure must be operating. Statistically significant differences in prevalence were noted, with the lowest prevalence at the highest altitude and the highest prevalence near sea-level (Walton et al., 1966).

As far as man is concerned, the only mode of infection apart from rare, accidental laboratory cases, cases from blood transfusions and organ

transplants, that is well-established is congenital. Since many animals whose flesh is used for meat by human beings, are infected, and since cyst form is resistant to digestion, it is likely that carnivorous and omnivorous animals and some humans acquire toxoplasmosis by consumption of raw or undercooked meat (Debrova, 1967; Remington et al., 1970a) and, possibly, even by handling of meat (Work, 1971). While there is little reason to doubt this mode of transmission to humans, it certainly cannot account for the high prevalence of infection among vegetarians or in herbivorous animals (Rawal, 1959). A breakthrough in the biology and epidemiology of toxoplasma seemed imminent following the important observation of the oocyst stage of T. gondii in the small intestines of the domestic cat, its excretion in the faeces and its ability to withstand environmental hazards (Frenkel et al., 1969; Sheffield and Melton; 1969; Hutchison et al., 1968; Work and Hutchison, 1969). Recently, Wallace (1971) demonstrated that oocysts of toxoplasma can be mechanically transmitted to human food by filth flies after their feeding on infected feline faeces. In addition, he observed that cockroaches could play the role of transport hosts for toxoplasma oocysts (Wallace, 1972a).

V. 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 Muhlpfordt (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.

Shettigara (1971) studied the growth characteristics of T. gondii (RH) in L, Vero and secondary chick embryo fibroblast (CEF) monolayers and observed that the rate and duration of growth of toxoplasma depended on the cell system used for its growth and on the input multiplicity of toxoplasma.

Ogunba (1972) has reported an infrequent observation of being able to locate intranuclear toxoplasma in the form of either rosettes or clusters of toxoplasma, in three of the seven cell lines investigated by him.

VI. PURIFICATION OF TOXOPLASMA FROM MOUSE PERITONEAL EXUDATE

Westphal (1958) employed ultrasonication to destroy host cells in the mouse exudate containing toxoplasma suspended in citrate buffer saline.

Niki (1959) and Tsunematsu (1960) ultrasonicated the suspension of toxoplasma and host cells and thereafter the suspension was digested by trypsin solution to destroy the partially injured host cells. However, trypsin is known to have a deleterious effect on toxoplasma, 0.5% trypsin solution killing the trophozoites completely, (Motomura, 1967) in 6 hours.

Lycke & Lund (1964a) purified toxoplasma from the mouse peritoneal exudate by centrifugation as follows: the suspension was initially centrifuged at 65 g for 5 minutes. Supernatant was taken and centrifuged at 440 g for 20 minutes and sediment was resuspended in medium and spun at 260 g for 10 minutes. Then the supernate was taken and centrifuged at 440 g for 20 minutes. They claimed to eliminate 90% of mouse cells and were able to recover 30% of organisms originally present in the exudate.

Fulton and Spooner (1960) used sintered glass filter of pore size 15-35 μ and anti red blood cell serum to remove contamination with the mouse cells. Neimark _et al._, (1967) and Remington _et al._, (1970b) employed a similar method as used by Fulton and Spooner for purification of toxoplasma.

Takeuchi (1971) reported that all the methods outlined above were carefully examined by him and were found unsatisfactory for the purpose of biochemical investigation. Filtrate contained host cells when they used sintered glass filter of pore size 15-35μ. Many host cells and red blood cells were found in the filtrate even when membrance filters of pore size as small as 8μ was tried. He purified toxoplasma trophozoites (RH) from the peritoneal exudate of experimentally infected mice using centrifugation with tris-sucrose-EDTA solution of specific gravity 1.072, and the combined use of the above-mentioned solution of specific gravity 1.074 and anti-mouse peritoneal cell serum plus antimouse spleen cell serum with a recovery rate of 25% and over 40% respectively. In both the methods, the elimination rate of host cells reached more than 99.9% in his hands.

Bodner *et al.*, (1972) reported of a cheap and least troublesome method of obtaining toxoplasma virtually free from all contaminating material by passing saline suspension of toxoplasma from mouse peritoneal exudate through a column containing Whatman CF 11 cellulose powder applying negative pressure. The effluent free from all of leucocytes was spun at 1,000 rpm for 30 seconds to remove erythrocytes. Further centrifugation at 1,500 rpm for 5 minutes resulted in obtaining the toxoplasma.

VII. 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, could be used for quantitative determination of the infectivity

of T. gondii and that the method was more sensitive and precise 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 visibly 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 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 millimeters in diameter. They have also reported development of clearly visible plaques on the chorioallantoic 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 CEF 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.

In his studies on in vitro neutralization by plaque assay in cell culture, Shettigara (1971) found in Vero monolayers a sensitive and reliable indicator system as it was found superior to L, secondary CEF and BHK cell monolayers.

VIII. 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 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 (IFAT) 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 0.2% 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 (1967a). 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 later work (Strannegard, 1967b) he reported to have obtained immune serum by bleeding rabbits eight days after an intravenous inoculation of about 50,000 toxoplasma parasites (apparantly living toxoplasma.) Suggs et al.,(1968) used toxoplasma grown in tissue culture, Ru-1 cells to immunize rabbits. The organisms were suspended in phosphate buffer solution (PBS), pH 7.2, and inactivated with beta propiolactone 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 comprising 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 sucutaneous route every alternate day. Freund's complete adjuvant was added in equal parts for the first injection.

Shettigara (1971) employed formolized toxoplasma (RH) to immunize rabbits, final concentraion of formalin being 1:4,000. Two injections were

given subcutaneously, the first injection comprising equal parts of formolized toxoplasma (obtained from the peritoneal exudate of infected mice) and Freund's complete adjuvant (FCA). Antisera against T. gondii (RH) was also prepared in rats and roosters with live toxoplasma obtained from infected mouse peritoneal exudate and suspended in PBS-A. Rats were injected subcutaneously as well as intraperitoneally and roosters, intramuscularly. Blood was collected by cardiac puncture from rabbits, rats and roosters.

Ourth (1971a) obtained peritoneal exudate of mice infected with T. gondii (RH) 3 days earlier, diluted it with an equal volume of sterile distilled water. It was then frozen at -80°C and then thawed in a water bath at 37°C ten times in succession. Equal volumes of this antigen and FCA were emulsified and 1 ml of the emulsion was injected subcutaneously into each of three rabbits, once a week for 3 weeks. Then five 0.5 ml injections of antigen only were given subcutaneously at one week intervals followed by 0.2 ml of antigen only subcutaneously each week for another 8 weeks and the rabbits were bled every week.

IX. 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 that had been previously tested and found to contain accessory factor. 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 (AMB) freshly prepared each time) is added and the proportion of

stained and unstained parasites is estimated in each dilution by examining a wet film under the light microscope. 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 AMB 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, in vitro cross neutralization tests using as antigens human and hare strains of toxoplasma and found that toxoplasma of hares appeared to be serologically identical with the human RH strain besides being equally pathogenic.

With a view to determine if various strains of T. gondii differed serologically, Wildfuhr and Hudemann (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 DT against 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. Apparently, the difference in the titres was not significant to classify them into different groups.

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 He La cells in Gey chambers. The effect of immune serum with and without AF was judged by the relative number of in-

fectious units, that is, the ratio between the number of parasites that penetrated the host cells and the number of exposed host cells, as observed under the phase-contrast microscope. 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 to his satisfaction. 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 further compared the neutralization test with DT and obtained parallel results. He concluded that they were detecting the same antibody.

In an interesting study Strannegard (1967b) reported his valuable 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 immuno-inactivation of toxoplasma by specific rabbit and human antibodies at various temperatures, 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 morphological alterations as observed under phase contrast microscopy.

The earliest work on neutralization may be credited to Sternberg (1892) who demonstrated that serum from a calf immunized against vaccinia was able to neutralize the vaccinia virus as evidenced when the immune serum-virus mixture, after allowing to stand at room temperature for an hour, followed by 24 hours in an ice-chest, was used to vaccinate a calf. On the other hand, normal calf serum failed to neutralize the virus under similar conditions. Fresh vaccinia lymph from an infected calf served as the source of vaccinia virus used in the experiments.

Early in the history of virology, Andrewes and Elford (1933a) presented one of the major principles of neutralization of viruses in the form of their theory of Percentage Law according to which, an approximately constant percentage of virus or phage is neutralized by a given dilution of antiserum regardless of the amount of added virus or phage, so long as antibody is in excess in the antibody-virus mixture.

Otto and Munter (1922) first recorded reactivation of neutral phage-antiphage mixtures by dilution. Burnet (1933) had obtained evidence pointing to dissociation of phage-antibody union in the presence of soluble substance derived from phage. This reactivation followed when highly

diluted serum was employed for neutralization of phage and then phage ultrafiltrate was added. There was no reactivation by simple dilution. David et al. (1953) reported that the infectivity of neutralized influenza A virus could be reactivated on dilution of the virus-antiserum mixture and tested in the mice. They were also able to demonstrate reactivation of neutralized influenza virus in the chick embryo on dilution of the virus-antiserum mixture. Further, they observed that the neutralizing titre of the antiserum, determined against a constant concentration of virus was dependent upon the host-cell system used as indicator system. A virus that appears to be completely neutralized, as judged by tests on one host cell system, may be highly infective in another cell system. (David et al., 1953; and Floorman and Trader, 1947).

Working with phages, Andrewes and Elford (1933b) put forward an attractive theory to explain the properties of incompletely neutralized phage. Accordingly, the surface of phage is believed to have active centres of two kinds - one with affinity for antibody (A) and the other with affinity for the host cell (B), the former determining the serological specificity of the phage and the latter the range of bacterial host species which the phage can attack. Neutralization of the antigenic (A) centres by antibody produces the condition of partial neutralization, the adsorption of additional antibody on to these centres may lead to the progressive blanketing of the adjacent (B) centres resulting in complete neutralization, accounting for the existence of a partly-neutralized phage as well as for the need for excess antibody to ensure complete neutralization of phage.

A possible method that may be used for the neutralization study of the various strains 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 (varying the serum: virus ratio) of neutralization, experiments to elucidate the effect of temperature, pH and ionic strength on the neutralization reaction. In addition, they demonstrated that the majority of virus particles constituting the persistent fraction was not genetically resistant to antibody as the progeny of the survivors in neutralization was found to be sensitive to antiserum when tested. Further, they concluded that the characteristics of the neutralization process were independent of the cell system used for assaying the surviving virus.

McBride (1959) demonstrated, employing 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, He La, and skin cultures were one and the same. He observed 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 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 degree 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 1965b; Taniguchi and Yoshino, 1965).

Studies with herpes simplex virus (HSV) 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). Their observations revealed that early 7 S and 19 S antibodies showed low to negligible neutralizing activity in the absence of complement or anti-gamma globulin. In the presence of complement, however, both these anti-bodies showed enhanced neutralizing activity. Further, the early 7 S but not the early 19 S antibody was capable of sensitizing the virus for subsequent neutralization by antigamma globulin. It is interesting to note that they found the late 19 S antibody could neutralize virus in the absence of complement or antigamma globulin and, in the presence of complement, the neutralization rate constant (K) but not the neutralization titres were enhanced. In contrast, the neutralization titres of the late

7 S antibody were enhanced approximately three-fold with antigamma globulin.

Laidlaw and associates (1935) carried out neutralization of a constant amount of influenza virus with serial dilutions of homologous antiserum prepared in horses and assayed on ferrets and laboratory mice separately. Using neutralization technique Magill and Francis (1936) demonstrated antigenic difference between various strains of influenza virus employing mice as the test animals. Employing the same technique Horsfall (1939) obtained a linear relationship between the logarithm of the quantity of antiserum and the logarithm of the quantity of influenza virus neutralized, enabling the determination of a fixed, rather than a relative, value for the neutralizing capacity of the antiserum.

Investigating further into neutralization of HSV sensitized with its homologous antibody in the Ig M fraction, Daniels _et al._, (1969) demonstrated that there was no neutralization of the virus in the presence of C'1; however, addition of C'4 in high concentrations brought about neutralization of the virus by its Ig M antibody. Further addition of C'2 and C'3 to the above mixture, did not enhance the neutralization titre. But in the presence of optimum concentration of C'1 and low concentration of C'4, addition of C'2 and C'3 resulted in enhanced neutralization. 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.

Shettigara (1971) carried out, amongst other things, _in vitro_ neutralization of _T. gondii_ (RH) in Vero monolayers by plaque assay against homologous antisera prepared by immunizing rabbits with formolinized toxo-

plasma and, rats and roosters with live toxoplasma and elucidated certain basic, yet, interesting aspects of neutralization carried out on similar lines as neutralization of viruses by various workers outlined above. Two methods of neutralization were employed: 1) standard method, in which, the reaction mixture was diluted 1:200 after appropriate neutralization time intervals in order to prevent further neutralization by the anti-serum, and 2) direct method, in which toxoplasma suspension initially taken in the reaction tubes was about 200 times less than the standard method and the reaction mixture was assayed directly without the 1:200 dilution being carried out. Vero monolayers were used as indicator system for assay of the surviving toxoplasma which were plaque-formers after one hour of neutralization.

The findings revealed in the above study, briefly, were as follows: rabbit and rat antisera required complement (guinea pig serum) to produce any neutralization by the standard method. In the direct method, however, rabbit antitoxoplasma serum did produce considerable neutralization in the absence of complement and enhanced neutralization in its presence. This was explained by the assumption that in the absence of complement, the binding of the rabbit antibodies to the antigenic determinants on the toxoplasma was weak and hence there was dissociation of the antigen-antibody complex during the process of dilution by a large factor, whereas complement, when added to the reaction mixture, helped to fortify the antigen-antibody binding which could, therefore, withstand dissociation on dilution. Rooster antisera, on the other hand, showed greater capacity for neutralization without complement than when complement was present in both the methods of neutralization. This observation was not very surprising as guinea pig complement was known not to activate avian anti-

sera (Brumfield et al., 1961; Benedict, 1967). The relatively less neutralization encountered in the presence of complement than in its absence is 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).

One of the striking features in the study was that the rabbit antiserum exhibited maximum neutralizing capacity when inactivated guinea pig complement was added and assayed by both the standard and the direct methods. Heat inactivation of guinea pig serum at 56°C for 30 minutes would result in the elimination of C'1, C'2 and C'5, the thermolabile components and perhaps to some extent, C'8 and C'9, the relatively heat-labile components of complement (Polley and Muller-Eberhard, 1966), as a consequence of which a decreased neutralization would be normally expected. But it is as well to remember that there are several inhibitors of complement, namely, 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). Components of guinea pig complements C'4 and C'6 are heat stable whereas inhibitors of C'4 and C'6 found in the guinea pig serum are heat-labile (Opferkutch et al; 1968; Tamura and Nelson, 1967). Inactivated guinea pig serum would therefore contain, among other components, C'4 and C'6 free from the inhibitory influence of their respective inhibitors to exercise neutralization effect on toxoplasma and this could account for the enhanced neutralization observed in the presence of inactivated complement.

It was also demonstrated that when both rooster and rabbit antisera, having identical IFAT-titres were tested separately, a higher percentage of neutralization was obtained with rooster antiserum than rabbit antiserum after one hour of neutralization. This could be explained by the difference in species from which antisera were obtained, but also suggests that IFAT was not detecting any or all of the components of neutralizing

antibody. Further, the fact that rooster antiserum is likely to consist of predominantly 19S antibodies rather than 7S antibodies (Uhr et al., 1962; Benedict, 1967) and the observations of Strannegard (1967a) that 19S antibody type is more effective per molecule than 7S antibody in the immunoinactivation of toxoplasma, could explain the above findings.

X. VIRULENCE

Virulence refers to the relative pathogenicity when a spectrum of microbial strains are compared in a given host under reproducible circumstances, and is related to both the invasiveness and the rate of multiplication (Kaufman et al., 1958). Only in experimental infections can the factors inherent in a strain, dose and route of inoculation be studied. Differences in virulence from strain to strain can only be studied in susceptible hosts using a "natural" mode of infection such as oral, intraperitoneal or subcutaneous, but not intracerebral since the results of intracerebral inoculation can be misleading (Frenkel, 1971).

Virulence is a measure of the ability of a living agent to overcome the body defences and cause disease, as well as of its invasiveness and toxicity (Peery and Miller, 1971).

Virulence of micro-organisms is defined as the capacity of the organism to invade the tissues, multiply and produce toxic effects (Cruickshank, 1965). It is estimated by the median lethal dose (LD50) for a number of individual animals of the same species and of uniform weight and age. Responses produced by different doses are observed and the dose that kills 50 per cent of the test animals within a specified time is taken as LD 50. The observations may involve a) the presence or absence of a

typical response, such as death or survival of the test animal. This "all or none" response is referred to in bioassay literature as a "quantal response"; or b) degrees of magnitude of response, such as time of survival of the test animal. This study of latent period or response time analysis is a quantitative response and is referred to in bioassay as a "graded response". Information on quantitative study relating time-to-death to the number of free organisms inoculated as well as LD 50 can provide a means for making comparative measurement of the virulence of the various strains of T. gondii (Eyles and Coleman, 1956).

Reports on LD 50 of toxoplasma have not been consistently uniform and some data are neither clear-cut nor unambiguous.

Jacobs and Jones (1950) found that 0.5 ml of 1:1,000 dilution of the peritoneal exudate from the intraperitoneally infected mice was infective for mice and they surmised that 5 toxoplasma would be a fair figure for inoculation by the intraperitoneal route to infect mice.

Chandler and Weinman (1956) figured out that a lethal dose for a 20 gm mouse was approximately 100 viable toxoplasma (apparently for RH and a porcine strain), death occurring about the ninth day.

Eyles and Coleman (1956) have given a brief account of a quantitative study on the relation of the number of toxoplasma inoculated to the time of death of the host as well as of the LD 50 inoculum. Their data were obtained by intraperitoneal inoculation of groups of mice with serum or saline suspensions of the peritoneal exudate of infected mice containing known number of toxoplasma trophozoites as obtained by counts in a haemocytometer. The LD 50 inoculum for organisms suspended in serum was found to be one toxoplasma compared with 30 toxoplasma when suspended in saline,

and the mean day to death of mice varied from 4-6 days to 9 days depending on the size of inoculum, the longest survival being 17 days among the four strains tested by them and observed for 20 days' period.

Chernin and Weller (1957) used toxoplasma from tissue culture and reported LD 50 to be 1 to 36 organisms.

Harboe and Erichsen (1955) have described a length difference between two strains of considerably divergent virulence and observed that the larger the toxoplasma the greater the virulence. However, they also found a number of intermediate strains, not easily distinguishable in this respect.

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.

Variation in virulence from strain to strain is a common phenomenon observed in T. gondii. Parasites isolated from severe cases of human toxoplasmosis are generally, though not always, highly virulent for mice and the strains isolated from latent infections in animals often tend to be of low virulence for mice, requiring numerous blind passages in mice before they can be demonstrable (Jacobs, 1956).

Jacobs et al., (1960) in their study found none of four ovine strains isolated was virulent for mice whereas seven of eight newly isolated porcine strains killed some or all of the mice inoculated with them and one

strain killed mice as rapidly as the RH strain. According to Meyer *et al.*,(1945) no appreciable loss in virulence occurred in a pathogenic strain maintained for a long period in tissue cultures.

Strains maintained in laboratory mice show increase in virulence not only for mice but for other hosts. The enhancement in virulence of an avirulent strain maintained in mice as compared with the same strain maintained in the yolk sac of chick embryo in which low pathogenicity is maintained has been described (Jacobs and Melton, 1954).

Kaufman *et al.*,(1958) have studied the growth of three strains of toxoplasma of varying virulence for mice in human amnion as well as monkey kidney cell cultures to observe that the virulent RH strain is capable of more rapid multiplication, invades a higher percentage of cells, and produces many more intracellular organisms than the moderately virulent 113 CE and the less virulent strain, S5. Kaufman *et al.*, (1958 and 1959) have shown that virulence in mice is correlated with greater invasiveness and more rapid reproduction in tissue culture. These findings have been confirmed by Hogan *et al.*, (1961) who studied the growth characteristics of five strains of toxoplasma in six cell lines and found that the virulence of an organism played an important role in the degree of cell destruction and the time of cyst formation; the less virulent strains produced less destruction in most cell lines with a tendency to produce cysts earlier. Strains of low virulence are usually good cyst-producers, but the activity of the parasite during chronic infection varies with the strain (Remington *et al.*, 1961). Marked strain differences in the rate of multiplication of organisms and in the rapidity of destruction of cultures were found by Kaufman *et al.*, (1958) in monkey kidney monolayer tubes.

Shettigara (1971) studied the growth characteristics of T. gondii (RH) in L, Vero and secondary chick embryo fibroblast cells to find that the rate and duration of growth of toxoplasma depended on the cell system used for its growth and on the input multiplicity of toxoplasma.

Motomura (1967) investigated the infectivity of T. gondii trophozoites (RH) and cysts (Beverley strain) for mice when administered by various routes. By the oral route (direct introduction into the stomach) 10^3 trophozoites were needed but only one cyst. The minimum doses to infect through skin abraded by sand paper were 5×10^3 trophozoites and 2×10^2 cysts; through deeply incised skin they were 15×10^2 trophozoites and 10 cysts.

Werner (1967) found that the ratio between the mortality rate produced by oral and by intraperitoneal administration to mice of cysts of weakly virulent T. gondii strains varied according to the virulence of the parasite. With parasites of low virulence, the oral route led to higher mortality; with strains of high virulence both routes were equally effective.

Csoka and Kulcsar (1968) reported that the LD 50 in mice for T. gondii (RH) was 100 and they therefore contended that the virulence of their strain remained high even after passage in primary human amniotic cells for eight months. It is not clear if the virulence of the strain was studied before serial passage in tissue culture. They obtained a LD 50 of 100 using only four groups of 12 mice, each mouse in the groups receiving 2,000, 1,000, 500 and 100 toxoplasma (RH), apparently intraperitoneally, and the death rates respectively being 100%, 100%, 100% and 50%.

The same authors in 1970 compared the growth of toxoplasma in primary human amnion cell cultures - the percentage of cells showing growth of toxoplasma against the time in days after inoculation with the mortality rate in mice and the time taken for their death. They concluded that the cell cultures proved equal or superior to mice in their sensitivity to toxoplasma.

In a comparative study of the sensitivity of the methods used for isolation of toxoplasma, Abbas (1967) found that mice intraperitoneally inoculated were at least 16 times as sensitive as chick embryos and at least 316 times as sensitive as HEp-2 and He La cells for isolation from acutely infected tissues. For isolation from chronically infected tissues, mice were 10 times as sensitive as chick embryos and the tissue cultures were of no value. He also confirmed the earlier findings of Jones et al., (1958) that the intraperitoneal route in mice was by far the best route for purposes of isolation of toxoplasma.

Passage of toxoplasma through the tick seems to decrease the virulence of the parasite but its original level of virulence is restored by one or two blind passages in mice. Virulence of toxoplasma appears to become enhanced on repeated passage in laboratory mice, occurring sometime precipitately and sometimes gradually. Relatively stable virulence appears to be maintained in tissue cultures or embryonated eggs (WHO, 1969).

XI. MOLECULAR FACTORS OF VIRULENCE

Virulence of strains of microorganisms cannot be fully explained in terms of known toxins and invasive mechanisms, the two factors which are associated with virulence but which may be largely independent of each

other, since strains which are endowed with the same known components may still vary greatly in virulence. This suggests that for each strain there may be some intrinsic factor contributing to its virulence (Cruickshank, 1965). Further, the capacity to invade cells and the capacity to multiply can be two different attributes of an organism. In toxoplasma, for example, pyrimethamine, aminopterin and sulfathiazole inhibited the growth or multiplication or reproduction of toxoplasma in He La cell cultures whereas none of these drugs affected the capacity of penetration or invasiveness of toxoplasma into the cells (Lycke and Lund, 1966).

Lycke et al., (1965) demonstrated enhancement of the penetration of T. gondii (RH) into He La cells by lysozyme and hyaluronidase.

In 1966 Lycke and Norrby demonstrated that lysed toxoplasma parasites contained factors promoting penetration of He La cells by toxoplasma. Supernatant of centrifuged preparation of lysed toxoplasma parasites when used for pretreatment of host cells, increased the relative number of infective units but no increase was noted after the pretreatment of toxoplasma.

From their studies of toxoplasma under electron microscope, Garnham et al., (1962) suggested an enzymatic activity for the paired organelles which are located at that end of the protozoan which attaches to and penetrates the cell wall.

Lycke et al., (1968) employed fractionation of extracts of disintegrated toxoplasma preparations in their study to find a direct correlation between penetration - enhancing effect of the preparations on the cell-penetrating capacity of the parasites and the increase in virulence for mice.

Lysosome-like structures of toxoplasma were observed by means of vital staining with acridine orange and by Gomori technique (Norrby et al., 1968). Before penetration, the parasites exhibited several lysosomes in the cytoplasm, whereas, shortly after penetration, the number of parasites with lysosomes was markedly reduced. The number of parasites with stainable lysosomes increased gradually after the host-cell penetration reaching a maximum at cell-burst.

Acid phosphatases and arylsulfatase have been detected in the cytoplasm of toxoplasma. Small amounts of lysozyme, hyaluronidase, β-galactosidase, and acid phosphatases have been demonstrated in various extracts of toxoplasma preparations (Lycke et al., 1968).

Toxotoxin was reported to be present in the peritoneal exudate of mice infected with toxoplasma by Weinman and Klatchko in 1950. It was reported as being heatstable and was lethal to mice by intravenous route. This was confirmed by Woodworth and Weinman (1960) who observed that the potency of the toxin was increased by heating at 56°C and that the toxin could be a protein of low molecular weight firmly attached to globulin carriers since it was nondialyzable. These findings were supported by Motomura (1966) who, in addition, reported successful preservation of the toxic activity of toxotoxin for 30 days at -4°C or -20°C and that the toxic substance was filterable in 0.45μ Millipore filter membrane.

Lunde and Jacobs (1962) have reported briefly on a toxin prepared by lysis of toxoplasma washed out of peritoneal exudates. This material contained, in contrast to toxotoxin, no mouse serum proteins and very few mouse inflammatory cells. The toxin was heat-labile, and resembled sarcocystin and Eimeria toxin in its activity in rabbits (Lunde et al., 1962).

Velasco and Varela (1966) employed permeable collodion sacs filled with the peritoneal exudate of infected mice for insertion into the peritoneal cavity of normal mice and found no evidence of symptoms attributable to toxotoxin. Fulton (1965) investigated extensively the toxic effect of peritoneal exudates from cotton rats infected with RH strain of T. gondii in mice by intravenous routes and concluded that similar toxic effects were noticed on intravenous injection of diluted calf synovial fluid or egg white and that T. gondii did not produce a true toxin.

Pettersen (1967) subjected toxoplasma lysate to column chromatography and then treating the eluate with rabbit antimouse serum, he was able to demonstrate that the toxic effect was non-specific and that it was caused by substances that originated in the mice.

In their study on the so-called toxotoxin, Nozik and O'Connor (1969) demonstrated that the lethal effects could be positively correlated with the viscosity and surface-tension properties of the peritoneal exudate. They attributed the lethal effects to protein-mucopolysaccharide complexes in the supernatant fluids from toxoplasma-induced mouse peritoneal exudates. However, recently Hogan et al., (1971) have reported to have obtained a purified preparation of toxotoxin by subjecting toxoplasma-infected peritoneal exudates of mice to ultracentrifugation, absorption with antimouse guinea pig spleen cells, and repeated lyophilization and dialysis against distilled water. It was finally suspended in distilled water as a clear, non-viscous sterile solution which, on injection into anterior chamber and vitreous of the eyes of rabbits produced necrosis of the vascular endothelium of the iris and ciliary body, destroying fibroblasts, melanocytes, neuronal and glial cells, a picture similar to the necrotizing

lesions seen in acute toxoplasmosis.

Detection of interferon in the sera after 8 hours following infection of mice with T. gondii, reaching a peak at 24 hours and persisting for at least 72 hours was reported by Rytel and Jones (1966). They could not detect interferon in culture fluids of mouse (L-929) cells infected with toxoplasma. Induction of interferon in the peritoneal fluid of mice infected with toxoplasma was demonstrated in vivo by challenging the mice with intraperitoneal injections of Mengo virus and in vitro in tissue culture with vesicular stomatitis virus (VSV) as the challenging virus after overnight contact of cell monolayers (cell of human origin-clone 1-5C-4(8) with the peritoneal fluid of infected mice, in both of which the protection afforded by interferon was significant. Characterization of the material was shown to fulfill many of the criteria used to define interferon.

Chick interferon prepared in embryonated eggs with influenza virus, protected CEF cells against VSV and toxoplasma infection and the mouse interferon prepared in L cells with Newcastle disease virus (NDV) against VSV and toxoplasma infections. Interferon preparations were uniformly more active in the virus assay than against toxoplasma (Remington et al., 1968). It is possible that the wide variation in virulence among different strains of toxoplasma may be related to their ability to induce interferon production in varying amounts.

Jones and Hirsch (1972) in their study of the interaction of T. gondii and mammalian cells such as fibroblasts, He La cells and mouse macrophages, combined cytochemistry and electronmicroscopy to observe changes in the

phagocytic vacuoles immediately following penetration by toxoplasma and was related to absence of lysosomal fusion and survival and multiplication of toxoplasma. The experimental evidence obtained by them suggested that about 50% of the toxoplasma which survived and thrived after penetration into macrophages, did so by being able to prevent the transfer of lysosomal materials to the phagosomes in which they were situated intracellularly.

XII. IMMUNODIFFUSION AND IMMUNOELECTROPHORESIS

In order to develop a more specific method for the aetiologic diagnosis of ocular toxoplasmosis in humans, the DT by itself being not satisfactory, O'Connor (1957a) made a study of the precipitating antibodies to *T. gondii*, employing (1) tube-dilution method and (2) agar gel-diffusion method of Ouchterlony. In the tube-dilution method, rabbit anti-toxoplasma serum having a DT titre of 1:50,000 or more was taken in serial dilutions in 0.85% sodium chloride solution in 0.5 ml volumes and to these freshly thawed toxoplasma antigen was added in 0.25 ml volumes, incubated at 37°C for one hour and at 7°C for 24-48 hours, whereas in control tubes in which non-infected mouse tissue protein (spleen tissue) was taken, no precipitate was seen. In the agar-diffusion method, lines of precipitation were observed after 24-48 hours in the cold when rabbit antiserum dilutions did not exceed 1:8. Gamma-globulin fractions from the same sera when tested with toxoplasma antigen gave identical lines of precipitation up to dilutions 1:32. When aqueous humour from patients suspected for ocular toxoplasmosis was tested in gel-diffusion, precipitin lines were observed with toxoplasma antigen but not with antigens from Staphylococcus, β-haemolytic Streptococcus, Histoplasma, purified protein derivative of

tubercle bacilli or non-infected mouse tissue protein, at a concentration of 1.0 mg antigen protein per ml.

In a second report O'Connor (1957b) used different preparations of toxoplasma antigen in agar-gel-diffusion against human and rabbit anti-toxoplasma sera and their gamma globulins to demonstrate one single dense band of precipitin common to all sera among a multiplicity of at least four lines. He was thus able to demonstrate precipitating antibodies to toxoplasma in the blood and aqueous humour of patients with ocular toxoplasmosis. In many instances precipitating antibodies were seen in aqueous humour but not in the blood, suggesting that antibody is formed locally in the eye. DT negative sera and sera with a DT titre of less than 1:256, did not give a positive reaction in agar-diffusion.

The antigen preparations consisted of 1) infected mouse peritoneal exudate diluted 1:2 in 0.85% sodium chloride solution and concentrated in a dialysis bag in a stream of cold air at 7°C for 5 to 6 days, 2) supernatant fluid dialyzed against a 5% solution of polyvinyl pyrrolidone for 24 hours, and 3) precipitation of mouse exudate with 1:1 cold saturated ammonium sulfate solution, centrifugation at 2,800 rpm for 20 minutes, resuspension in distilled water and dialysis against tap water overnight and against 0.85% sodium chloride solution at 7°C for three days. The soluble and insoluble fractions were tested separately for antigen potency. The supernatant fluid from the peritoneal exudate of infected mice gave more satisfactory results. Dialysis against polyvinyl pyrrolidone resulted in apparent denaturation of antigen. Antisera and their gamma globulins gave identical results. Double agar gel-diffusion study was also used for the analysis of autoradiographic detection of toxoplasmic antibodies by O'Connor (1957b).

Korting (1958) analyzed precipitating toxoplasma antigen-antibody systems with the aid of immunodiffusion and immunoelectrophoresis. He employed dried toxoplasmin as antigen and obtained precipitin lines with rabbit antisera which had high DT titres. The electrophoretic separation of the immune serum showed that the toxoplasma antibodies migrated with the gamma globulin fractions of rabbit serum. Only one precipitation line with a sharp edge on one side was produced. Toxoplasma antigen showed components with different electrophoretic migration velocities, forming at least four separate precipitin lines with the homologous antibody fraction. They were situated in the albumin, α1-, α2-, and β2-regions possibly a fifth component in β2- to γ- region.

In his studies on the relationship between the toxoplasma antibody components demonstrated by the DT, complement fixation test (CFT) and gel-diffusion techniques, Strannegard (1962) did not find good correlation between either the DT titre and the precipitin bands or the CFT titre and the precipitin bands. In his investigations he obtained two lines of identity between human and rabbit antisera; more precipitin lines appeared with rabbit sera than with human sera. When rabbit antiserum was obtained by repeated injections of formalin-killed toxoplasma, one or two faint lines of precipitation were obtained in agar-diffusion but not until three months had lapsed after the beginning of the injection schedule. Study on the dynamics of antibodies in rabbits revealed that the precipitins seemed to appear later during the disease than DT - and CFT - antibodies. Electrophoretic analysis of toxoplasma antibodies showed that the DT- and CF- antibodies of human sera had a mobility similar to human serum β-2 globulins. Precipitins of human sera seemed to be γ-globulins with small numerical mobility.

The following antigen preparations were employed by Strannegard in the gel-diffusion study. 1) Sediment: "72 hours" infected mouse peritoneal exudate was collected, heparinized (50 IU heparin/ml) and centrifuged. The sediment was suspended in physiological saline, frozen and thawed five times, homogenized and then again frozen and thawed five times. 2) Supernatant: supernatant from centrifuged mouse exudate was frozen and thawed five times, concentrated 5 to 10 times by pervaporation in dialysis tubing at room temperature. 3) Ultrasonic treatment: Peritoneal exudate, sediment and supernatant were separately subjected to ultrasonic treatment at frequencies of 200 kHz and 1.6 MHx for different periods of time. 4) Distilled water lysed sediment.

Antigenic preparations from chorioallantoic membrane of chick embryos which had been inoculated 5 days earlier were used after freezing and thawing 8 times, followed by grinding in a mortar and resuspending in physiological saline and freezing and thawing again 8 times. The suspensions were centrifuged and the supernatants concentrated 5 to 10 times by pervaporation in dialysis tubing. This antigen gave, as a rule, less numerous, often fuzzier lines than did antigens from mouse exudate, namely, the sediment and the supernatant; the latter in particular gave the maximum number of precipitin lines. Distilled water lysed antigen did not give so many precipitin lines as sediment or supernatant and ultrasonic treated antigen did not give more lines than supernatant antigen.

Subjecting the sediment antigen to various temperatures for 20 minutes, Strannegard was able to obtain four precipiting lines with the antigen subjected to 70°C, 2 lines at 90°C, 1 line at 100°C and no lines at 120°C, whereas at room temperature the antigen gave 5 precipitin lines with the homologous antiserum.

Spano and Dardanoni (1969) were able to conclude that there were distinct, heat-labile and heat-stable antigenic components in T. gondii (RH) as a result of their study with three human antitoxoplasma sera before and after absorption with unheated and heated (100°C for one hour) toxoplasma sediment in the dye tests and in indirect fluorescent antibody tests. Heated toxoplasma were slightly active in immunofluorescent tests with unabsorbed sera.

Sera and aqueous humour from 28 patients were tested for the presence of precipitating antibodies by Kaufman (1960) by agar-diffusion method described by O'Connor.

Hubner and Uhlikova (1969) employed agar gel precipitation test using lyophilized supernatant from infected mouse peritoneal exudate as antigen in their diagnostic study of toxoplasmosis and reported perfect correlation with isolation of toxoplasma as well as DT results. Microprecipitation in agar (MPA) using heparinized whole blood instead of serum gave equally satisfactory results in their hands. The number and intensity of the precipitation lines was not influenced either by prestorage of sera in a refrigerator or by inactivation. Lyophilized supernatant used as antigen can be kept frozen for at least sixteen months without loss of activity.

In a subsequent study, Hubner and Uhlikova (1970) investigated the correlation of MPA and DT with the results of isolation of toxoplasma from the brains of various animals such as rabbits, cats, chicken, duck, guinea pigs etc. Mice were used for isolation tests. Out of a total of 72 animal sera belonging to ten species tested by MPA and DT and the brains used for isolation, 26 sera were negative in both tests, in 26 the DT only

was positive and in 20 both reactions were positive. T. gondii was successfully isolated in 18 cases, in all of which the sera were MPA positive.

MPA was positive in 90% and DT in only 39%. The authors concluded that MPA reaction indicated the presence of living toxoplasma organisms in the host animals; in other words, MPA indicated non-sterile immunity. In support of this theory, they claimed that MPA gave negative results unlike DT when sera from a group of mice immunized with a single dose of dead toxoplasma antigen prepared by multiple freezing and thawing of the toxoplasma sediment in white mouse peritoneal exudate and then dissolved in saline. Whereas DT reached maximum value between the first and second week after inoculation and remained constant between 1:4 and 1:64 till the end of observation period of one year, MPA was found negative throughout.

Recently, Ourth (1971a) demonstrated only one precipitin band under immunoelectrophoresis (IEP) allowing rabbit antitoxoplasma serum absorbed with dried normal mouse serum (NMS) to electrophorese and then to react against toxoplasma antigen. The single band obtained was approximately in β-2 position. These results, obtained by absorption with NMS and IEP, would seem to indicate to them that T. gondii produced one parasite band. The antigen used for immunization was ten times frozen and thawed peritoneal exudate of mice infected with T. gondii (RH). The exudate was diluted with an equal volume of sterile distilled water.

Employing 40 precipitin bands that were washed and homogenized and emulsified with FCA to produce antiserum in rabbits, they obtained a single precipitin in IEP against the antigen. However, the precipitin arc pro-

duced with the monospecific antibody was slightly longer than that produced with absorbed antiserum. In a subsequent study, Ourth (1971b) conjugated the toxoplasma monospecific antibody with fluorescine isothiocyanate and employing fluorescence antibody technique successfully demonstrated toxoplasma cysts in pepsin-digested tissues of mice infected with a human strain of T. gondii cyst.

XIII. POLYACRYLAMIDE-GEL ELECTROPHORESIS

In an attempt to elicit strain differences of T. gondii, Bloomfield and Remington (1970) made a study of proteins from three strains by means of acrylamide-gel electrophoresis at pH 8.3 and 4.5. When the electrophoretic patterns were compared for differences in either electrophoretic mobility of certain proteins or in the number of bands, no significant differences were demonstrable.

XIV. RADIOIMMUNOASSAY

Radioimmunoassay (RIA) was originally introduced by Berson and Yallow (1968). Various radioimmunoassays have been since described and many of them have found application in routine clinical laboratory diagnostic tests. RIA has the advantage over other clinical procedures in having an extremely high sensitivity, being able to measure antigenic concentrations in the range of micro, nano-, or picograms (Skelley, et al., 1973). Non-specific binding of antigen to serum proteins other than antibody can be determined by running a blank with each assay.

Radioimmunoprecipitation (RIP) popularly employed in diagnostic viral serology, generally involves incubation of labeled virus with dilutions of antiviral antibody and precipitation with anti-immunoglobulin. By measuring the

amount of radiolabeled virus precipitated, the titer of antiviral antibody is determined (Rosenthal et al., 1973). However, most of these assays require highly purified antigens or antibodies. This problem can be overcome by using radioactive antiglobulin sera which is highly specific and can be purified and employed with advantage in the indirect RIA.

Harder and McKhann (1968) originally described an indirect isotopic antiglobulin test for the detection of tumour-associated cell surface antigens in murine SV-40 and Polyoma tumour cell surface antigens in cell suspensions. Recently solid-phase assays have been increasingly used to detect viral antigens and measure antiviral antibody. Techniques of bonding of antigen or antibody to a solid phase make use of antibodies covalently bonded or fixed to insoluble polymers (immunosorbents) such as bentonite particles, cross-linked dextrans (Sephadex), beaded agarose (Sepharose) or bromacetyl cellulose, or covalently cross-linked to one another with ethyl chloroformate or glutaraldehyde or physically adsorbed to a polypropylene or polystyrene plastic tubes (Skelley et al., 1973). Burdick and associates (1973) described an indirect micro-solid phase RIA using tissue culture monolayers and radioactive antiglobulin for detecting methyl-cholanthrene-induced tumour specific surface antigens. The cell monolayers employed by them allowed a simple washing and yielded sensitive results.

A comparative study on direct and indirect solid-phase microradioimmunoassays for detecting viral antigens and antiviral antibody was made by Rosenthal and co-workers (1973) who found the indirect method far more sensitive than the direct method, presumably due to attachment of more than one molecule of labeled antiimmunoglobulin to each molecule of un-

labeled antiviral antibody. The amount of antiviral antibody bound to viral antigens was determined by them by measuring the extent to which the antiviral antibody inhibited the specific binding of ^{125}I-labeled antiviral IgG in the direct technique or enhanced the specific binding of ^{125}I-labeled anti-IgG in the indirect technique. They observed that a high concentration of proteins in the diluent used with the viral antigens decreased specific binding, whereas a high concentration of protein in the diluent used with ^{125}I-labelled antiIgG increased specific binding by decreasing non-specific attachment of labeled antiIgG.

MATERIALS AND METHODS

NEUTRALIZATION STUDY

Sources of toxoplasma strains:

Strain of T. gondii	Source of Origin	Supplied by:
RH	Isolated from an encephalitic child by Sabin in 1941	Dr. Walls, K., CDC, Atlanta, Georgia
V16	Isolated from chicken in Iowa in 1965.	Dr. Walls, K, CDC, Atlanta, Georgia
C56	Isolated from chicken at the National Institute of Health, Maryland	Dr. Sheffield, Maryland
Beverley	Isolated from rabbit by Beverley in UK.	Dr. Sheffield, Maryland
C37	Isolated from chicken.	Dr. Remington, California

The above five strains of T. gondii were received either in the form of infected mouse brains or suspended in heparinized blood. Immediately on receipt of the materials, each material was injected intraperitoneally into an average of six Swiss White mice known to be free from T. gondii infection as their sera revealed no titres on IFAT for toxoplasma antibodies. While blood samples were injected directly into mice in 0.5 ml volumes per mouse, infected mouse brain specimens were homogenized in a small aliquot of sterile physiological saline (plus antibiotics) and the saline suspension was injected in 0.5 ml amounts per mouse. Each strain was subpassaged several times in mice until the strain was "fixed", that is, until the infected mice became morbid at regular intervals. For example, with RH and V16 strains, mice injected intraperitoneally with 0.1 to 0.2 ml of a 1-10 mouse exudate-saline suspension, regularly became morbid in 3 or 4 days, although in the initial passage it took 8 or 9 days for mice to become morbid, whereas with C56 strain, it was about 15 days for the initial

passage and it took several passages before the organisms regularly killed mice in 4 to 5 days.

While each strain of toxoplasma was maintained in Swiss White mice by serial passages, once the strains were "fixed", an aliquot of each strain of toxoplasma suspension from the mouse exudate was mixed with an equal volume of "freeze mixture" containing 20% dimethyl sulfoxide (DMSO) (Shettigara, 1971), distributed in one ml volumes 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).

Preparation of parasite suspension:

Each strain of toxoplasma was maintained in Swiss White mice of six to twelve weeks' age, weighing 20 to 28 grams. 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 sterile physiological saline solution (plus antibiotics), 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 3 or 4 days for the purpose of maintenance of the strains of toxoplasma for use in the experiments.

Toxoplasma strains for neutralization experiments were collected in the buffer stated under the experiments and for virulence tests, the collecting fluid was PBS-A with 2% heat-inactivated calf serum. The collecting fluid was taken in a sterile stoppered glass tube with sterile glass beads

and to this 200 units/ml penicillin, 200 μg/ml streptomycin and 2.5 μg/ml amphotericin B (Fungizone) (PSF) was added. The glass tube was kept in ice bath at all times. After rolling with glass beads for about 3 minutes, to defibrinate, an approximate count of live, extracellular toxoplasma was made in a Nebauer-Levy haemocytometer after a 1-10 dilution of toxoplasma suspension in AMB dye solution in a separate test tube.

For neutralization and virulence experiments, the mice were infected with about 5 to 10 times the above dosage of toxoplasma intraperitoneally. RH and V16 strains were collected 48 to 60 hours following inoculation and C56, C37 and Beverley strains 60 to 72 hours after inoculation.

Preparation of purified toxoplasma suspension:

Toxoplasma from infected mouse peritoneal exudate was collected in Hanks's balanced salt solution (HBSS) pH 7.7 following the same procedure outlined in the preparation of parasite suspension. The suspension was initially centrifuged at 65 g for 10 minutes and the sediment was discarded. The supernatant was centrifuged at 650 g for 20 minutes and the sediment was reconstituted in a small aliquot of the buffer to which PSF was added in the concentrations given above and count of live, extracellular toxoplasma was made as stated already before using in the neutralization experiments.

Preparation of hyperimmune antiserum in rabbits:

Two rabbits were immunized by subcutaneous injections of formolized T. gondii of each strain. Toxoplasma obtained from the peritoneal exudate of 3 to 5 Swiss White mice infected 72 hours earlier was collected in a sterile glass bottle containing 5 to 8 ml PBS-A with sterile glass beads,

rolled for a few minutes to defibrinate and an approximate count of toxoplasma in the suspension was made under haemocytometer after staining with AMB. The suspension was spun down at 650 g for 20 minutes and the sediment was suspended in the appropriate amount of PBS-A to obtain about 10×10^6/ml of toxoplasma. To nine parts of this suspension one part of 1-400 formalin (diluted in PBS-A) was added to make a final concentration of 1-4000 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.

Each rabbit received seven injections, on the days stated in the schedule (Table 1). First injection consisted of 0.5 ml each of FCA and formolized toxoplasma suspension containing about 5×10^6 toxoplasma, given subcutaneously at five different points at the upper nuchal region. Later injections comprised 0.5 ml of formolized toxoplasma suspension only. Test bleeding was made a week after the final injection and 20 to 30 ml of blood was collected from the ear vein. The rabbit was bled similarly twice or thrice at 3-day intervals and was finally bled by cardiac puncture under ether anaesthesia. Blood was always collected in sterile centrifuge tube, allowed to clot at room temperature for about an hour, and was centrifuged at 2,400 g for 30 minutes. Serum was decanted into another sterile centrifuge tube which was spun at 1,000 g for 20 minutes. All the sera samples in respect of each strain were pooled, sterility tested in AC medium* and Sabouraud's broth*. In the event of any contamination, the serum was filtered through Millipore membrane of 0.3 μ pore size, tested for sterility as before and kept at -20°C.

* Difco Laboratories

Three days prior to immunization, each rabbit was bled by the ear vein for collection of preinoculation serum.

All preinoculation and hyperimmune sera were inactivated at 56°C for 30 minutes just before 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 IFAT and were found negative.

Preparation of early antiRH serum in rabbits:

T. gondii (RH) was purified as stated earlier using PBS buffer, pH 7.0 instead of HBSS. Toxoplasma thus obtained was formolized at a final concentration of 1-4,000 formalin. After allowing to stand at room temperature for one hour and a fortnight at 4°C, it was washed twice in PBS, pH 7.0 and was finally suspended in PBS. Rabbit was given 0.5 ml of this suspension intravenously in 0.5 ml volumes, containing about 5×10^6 toxoplasma, on day 1, 2, 3, and 4, and was bled by the ear vein on day 7, 8, and 9. Sera collected from these bleedings were pooled, sterility tested and kept at -20°C.

Concentration of antisera:

Each of the five strain specific rabbit antitoxoplasma sera as well as preimmune rabbit serum was separately taken in 60 ml volumes in a dialysis tubing 25/64*. The dialysis tubing was first boiled in distilled water containing 3% acetic acid for a few minutes, rinsed with distilled water and then used. The tubing containing the serum was placed inside a 4 litre flask with a side tubing. Negative pressure was applied gently

* Union Carbide Canada Ltd.

TABLE 1

SCHEDULE OF INJECTIONS GIVEN FOR PREPARATION

HYPERIMMUNE ANTISERUM IN RABBITS

Week	Day of the Week		
	Monday	Wednesday	Friday
First	1st inj.	2nd inj.	3rd inj.
Second	———	———	———
Third	4th inj.	5th inj.	6th inj.
Fourth	———	———	———
Fifth	———	7th inj.	———
Sixth	———	——*——	———

* Test bleeding of 20 to 30 ml.

and the flask was transferred to the cold room (4^0C). When the volume of the serum was reduced to about a third of its original volume, the pressure was slowly released and the serum was dialyzed against physiological saline for 48 hours in the cold. The dialysate in two litre volumes was changed at 24 hours. Concentrated serum thus obtained was filtered through a Millipore membrane of 0.3μ pore size, tested for sterility and kept at -20^0C.

Tissue culture: (Vero cell monolayers):

Vero cells* had been obtained through the courtesy of W. Stackiw, Manitoba Provincial Virology Laboratory and kept frozen in one ml ampoules at -140^0C. Monolayers were obtained from the contents of frozen ampoules and subcultured every 3 or 4 days. Monolayers in small Povitsky bottles@ were treated with about 25 ml of either a mixture of 1-5,000 ethylenediaminetetraacetic acid (EDTA) plus 0.05% trypsin in PBS (pH 7.3) or with 0.25% trypsin in HBSS pH 7.3, for 5 to 15 minutes at 37^0C, centrifuged at 160 g for 7 minutes and the cell pellet 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 dish# and the petri dishes were incubated at 37^0C in a humidified atmosphere of 5% CO_2. Invariably confluent monolayers were formed after 48 hours incubation.

Whenever the plaque assay was carried out in T-flasks#, they were incubated at 37^0C in an ordinary incubator. Vero monolayers were similarly obtained as in petri dishes except that the growth medium used was minimum essential medium (MEM) with 10% calf serum and PSF, instead of RIF growth medium employed for monolayers in petri dishes.

* Vero cells were originally derived from the kidney epithelium of an African green monkey (Cercopethicus aethiops)
@ Kimble glass, two litre capacity.
\+ Growth medium was RIF (See Appendix A for composition)
\# Falcon plastics, a division of Becton, Dickinson and Co.

Complement:

Guinea pig serum* was distributed in 0.5 ml amounts in sterile stoppered glass tubes in Revco at -87°C after filtration through 0.3 μ Millipore membrane. Prior to use, the tubes were immersed in a water bath at 37°C and thawed quickly. Whenever heat inactivated complement was used, it was inactivated at 56°C for 30 minutes just prior to use in the experiment. Dilutions of complement to the required titre was done using cold, sterile physiological saline. Every six months guinea pig serum was obtained from the same source and complement titrations carried out as before.

Plaque Assay:

Plastic petri dishes,+ 60x15 mm were used in the plaque assay and neutralization by plaque assay experiments. The dishes were seeded with five ml growth medium containing approximately 2 x 10^5 Vero cells/ml. The cells were allowed to form a confluent monolayer at 37°C in a humidified atmosphere of 5% CO_2 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 suspension was used as the inoculum, it was specifically stated under the experiment. The petri dishes were incubated at 37°C for two hours to allow adsorption of toxoplasma, at the end of which period, the monolayers were overlaid with five ml agar overlay. 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% CO_2 in air. A second overlay of nutrient agar (See Appendix) in 5 ml amounts per petri dish was added on the sixth day and further incubated after allowing it to

* Complement was obtained from Mr. W. Stackiw, Manitoba Provincial Virology Laboratory and it was titrated in Dr. Sayed's Laboratory, Med. Microbiology, following the procedures outlined in Kabat and Mayer (1961).

\+ Falcon Plastics, a division of Becton, Dickinson & Co.

solidify. On the 11th day, three ml neutral red agar was applied to each plate, allowed to solidify at room temperature, incubated overnight, allowed to stand in the dark at room temperature for 12 to 24 hours and the plaques which were about 2 mm in diameter were counted over a light-box.

Plaque assay in T-flasks in ordinary incubator:

The procedure was the same as with petri dishes except that 6.0 ml nutrient overlay was applied after allowing toxoplasma to adsorb and after the agar solidified, the T-flasks were turned over and incubated at 37°C in an ordinary incubator for six days. On the seventh day 2.5 ml neutral red agar was added, allowed to solidify, and then kept in the incubator overnight. The flasks were kept in the dark at room temperature for 12 to 24 hours and the plaques which were about one millimeter in diameter were read using a magnifying lens (7X magnification).

Neutralization Technique:

The neutralization technique applied in the study was essentially based on that described by Dulbecco et al., (1956), McBride (1959), and Ashe and Scherp (1963). Antitoxoplasma serum (strain specific) whose titre had previously been determined by IFAT, was distributed in 0.5 ml amounts in sterile stoppered glass tubes. Throughout the experimental study, toxoplasma was obtained from the peritoneal exudate of Swiss White mice infected 48 to 60 hours earlier in the case of RH and V16 strains and 60 to 72 hours earlier in the case of C56, C37 and Beverley strains. Unless otherwise stated, toxoplasma suspension was diluted with HBSS, pH 7.7, so

as to contain approximately a known number of live, extracellular toxoplasma (usually 4×10^6/ml) as determined by count in haemocytometer after staining with AMB stain (see Appendix). Toxoplasma suspension was taken in 0.5 ml amount in separate sterile, stoppered glass tubes. Whenever complement or inactivated complement was used it was added in equal volume (0.5 ml) to toxoplasma suspension. To this, 0.5 ml of buffer was added. At least five minutes before mixing, the tubes were separately kept in a water bath at 37°C and thus prewarmed to 37°C. At time zero and at appropriate intervals thereafter (as given under each experiment), 0.5 ml of the mixture was pipetted into 100 ml of cold diluent (PBS-A with 2% calf serum, plus PSF) in order to prevent further neutralization. Of this diluted sample 0.5 ml was assayed for surviving toxoplasma on each of two replicate Vero monolayers, allowing two hours' adsorption time before the addition of five ml of nutrient agar per plate. Whenever there were changes in the volumes of components of reaction system or in the concentration of toxoplasma suspension, they have been stated under the experiment.

Control tubes contained, instead of antiserum, equal amount of rabbit preimmune serum or normal rabbit serum known to have been negative in IFAT.

In the direct method, toxoplasma suspension was initially diluted to contain about 2×10^4/ml and 0.5 ml or the appropriate amounts as given under the experiment was taken in each tube. This was the only change in the reaction system and the surviving toxoplasma were assayed for plaques at appropriate time intervals directly from the mixture to the Vero monolayers without carrying out 200-fold dilution.

In experiments with purified toxoplasma, 0.2 ml each of toxoplasma suspension at a concentration of about 5 x 10^4/ml, buffer, inactivated complement and serum were taken and assayed directly on Vero monolayers. In experiments with varying concentration of purified toxoplasma, the concentration of toxoplasma was proportionately greater according to the magnitude of the dilution factor, and is given under the pertinent experiments.

VIRULENCE STUDY

Virulence tests:

For the determination of fifty per cent lethal dose (LD50) of each strain of *T. gondii* in terms of plaques and for the determination of average response time (RT50), Swiss White mice of both sexes in the age range of about six to twelve weeks and weighing between 20 and 28 grams were used. Toxoplasma obtained from the peritoneal exudate of mice infected 48 to 60 hours earlier in respect of RH and V16, and 60 to 72 hours earlier in respect of C56, C37 and Beverley strains, was collected in PBS-A with 2% calf serum and PSF with sterile glass beads in a sterile stoppered glass tube. The tube was kept in ice bath at all time. After rolling with glass beads for about 3 minutes to defibrinate, an approximate count of live, extracellular toxoplasma was made as stated in the preparation of parasite suspension. Starting at a concentration of 1 to 2 thousand/ml, serial two-fold dilutions were made using the same buffer as diluent. In all about ten to thirteen serial dilutions were used for plaque assay on Vero monolayers in 60 mm petri dishes and the last seven

to ten dilutions were used for injecting the mice. 0.5 ml from each dilution was inoculated into each Vero monolayer, five such monolayers in petri dishes being used for each dilution and assayed for plaques as described earlier in plaque assay. A group of ten mice selected at random was used for each dilution, each mouse receiving 0.5 ml of the suspension intraperitoneally under ether anaesthesia. As controls, a group of ten mice was injected intraperitoneally with 0.5 ml/mouse of PBS with 2% calf serum plus PSF, and another group of ten mice was kept uninoculated. All the mice were observed daily for any death up to 20 days. Death among the injected mice occurred between eight and sixteen days, both days inclusive. No death was observed after sixteen days' survival.

Record of the number of mice that died in each group of mice was made day-wise.

The preparation of various dilutions of toxoplasma for inoculation into Vero monolayers was accomplished in about one hour after the peritoneal exudate was withdrawn from the mice. Intraperitoneal injection into mice took about another one hour.

At least two experiments were carried out for each strain of <u>T. gondii</u>. From the data obtained of the average number of plaques for each dilution and the corresponding percentage of mortality among mice, LD50 in terms of plaques as well as its standard error was calculated applying probit transformation as detailed under statistical analysis. RT50 values were obtained

by rankit transformation of the times-to-death of mice under each dilution. Both LD 50 and RT 50 values in respect of each strain of toxoplasma were made use of in determining the virulence of each strain.

Plaque assay (vide pp 51 under Neutralization study)

IMMUNODIFFUSION AND IMMUNOELECTROPHORESIS STUDY

Preparation of toxoplasma antigens for immunodiffusion and immunoelectrophoretic studies:

1. From Mouse Exudate -

a) Distilled water lysed antigen - Peritoneal exudate from 8 to 10 mice infected with each strain of *T. gondii* 72 hours earlier was collected separately in PBS-A with sterile glass beads in a sterile stoppered glass tube, shaken well for about 3 minutes and then centrifuged at 65 g for 7 minutes. Supernatant was decanted and spun at 650 g for 20 minutes. Pellet was washed twice with PBS-A, resuspended in about 10 ml of distilled water, allowed to stand at 4°C overnight. It was then lyophilized and the dry powder was resuspended in 2 ml physiological saline.

b) Sediment - Peritoneal exudate from 8 to 10 mice infected with each strain of toxoplasma as above was similarly collected separately in physiological saline and centrifuged at 650 g for 20 minutes. The sediment obtained was suspended in about 2 ml physiological saline in a stoppered glass tube. It was then frozen in dry ice-alcohol mixture for about 15 minutes and thawed at 37°C in a water bath quickly. Freezing and thawing was repeated four more times.

c) Supernatant - Supernatant obtained after collecting the sediment in the above procedure, was frozen and thawed five times as above, lyophilized and the dry powder was suspended in distilled water to about 1/5th the original volume.

2. From Tissue Culture

a) Tissue culture antigen - Each strain of toxoplasma obtained from the peritoneal exudate of mice infected 72 hours earlier was separately collected in PBS-A with sterile glass beads, shaken for about 3 minutes, and centrifuged at 65 g for 7 minutes. Supernatant was collected and spun at 650 g for 20 minutes. The sediment was washed 2 times with PBS-A, and resuspended in PBS-A. Two ml of this suspension containing about 5×10^6 toxoplasma/ml of each strain was separately inoculated into two prescription bottles each, containing Vero cell monolayers, allowed to adsorb for two hours, washed twice with MEM and then 25 ml of MEM with 10% calf serum was added and incubated at 37°C. The medium was replaced every alternate day three times and from then onwards the medium was collected every other day, centrifuged at 650 g for 20 minutes and the sediment was suspended in a small aliquot of distilled water in a sterile test tube. After collecting 3 to 4 times as above, the antigens were frozen and thawed five times, lyophilized and the powder obtained was resuspended in a small amount of physiological saline. The undissolved particles were allowed to settle down the tube and the supernatant was used as antigen.

Preparation of tissue culture control antigen:

Mouse peritoneal exudate from six Swiss White mice injected intraperitoneally with sterile dextran* in 1.5 ml amounts per mouse three days earlier was collected aseptically in PBS-A with sterile glass beads in a sterile stoppered bottle, centrifuged and washed similarly as the exudate containing toxoplasma for tissue culture inoculation for the preparation of tissue culture toxoplasma antigen. Inoculation of the Vero monolayers in prescription bottles with the control material, adsorption time of two hours, followed by washing and addition and replacement of growth-medium were all carried out exactly the same way as with the preparation of tissue culture toxoplasma antigen. The medium was similarly collected and treated similarly as the test antigen to obtain the control antigen.

Preparation of normal mouse peritoneal exudate:

Six Swiss White mice were each injected intraperitoneally with 1.5 ml of dextran* (5 to 50 million molecular weight, 35 mgm/ml in PBS, autoclaved and kept frozen). After three days peritoneal exudate was collected in a small aliquot of PBS-A with sterile glass beads, shaken for about 3 minutes, and centrifuged at 650 g for 20 minutes. The supernatant was frozen and thawed five times, lyophilized and the dry powder was reconstituted in distilled water to about 1/5th the original volume.

Preparation of hyperimmune antiserum in rabbits (vide pp 46 under Neutralization study)

* Dextran was obtained from Dr. I. Berczi, Department of Immunology, Medical College, Manitoba, Winnipeg

Absorption of antiRH and antiV16 sera with toxoplasma antigens:

Each strain of toxoplasma from the peritoneal exudate of mice infected 72 hours earlier was collected separately in physiological saline with sterile glass beads, rolled for a few minutes, centrifuged at 65 g for 7 minutes, supernatant collected and centrifuged at 650 g for 20 minutes and the pellet of toxoplasma obtained was used to absorb an equal volume of antiRH serum which was mixed well with the pellet of each strain in separate test tubes, allowed to stand at room temperature for one hour and overnight at 4°C and then centrifuged at 1000 g for 30 minutes and the supernatant was collected and kept at -20°C until used in the experiments. Similarly, antiV16 serum was absorbed with RH strain of toxoplasma in the ratio of 4:1, 3:1, 2:1, and 1:1.

Absorption of antisera with normal mouse serum powder:

Method 1. Normal mouse serum (NMS) was distributed in 0.5 ml amounts in several test tubes, frozen in dry ice-alcohol mixture and lyophilized. To each tube containing dry NMS, 1.5 ml, 1.0 ml, 0.5 ml, and 0.25 ml of antiRH serum was added so that the ratio of antiserum to NMS was 3:1, 2:1, 1:1, and 1:2 respectively, well mixed and allowed to stand at room temperature for one hour and overnight at 4°C. The mixture was centrifuged at 3020 g for 30 minutes and the supernatant was used in the experiments. Similarly other strain specific antisera were absorbed with NMS powder in the ratio of 1:1 and 1:2.

Method 2. NMS protein(s) was linked with glutaraldehyde for absorption of antibodies to mouse protein(s) by the method of Avrameas and Ternynck

(1969) with modifications. To 2.0 ml of NMS which was dialyzed overnight against 0.85% NaCl solution in the cold, 0.2 ml of 1 M acetate buffer (pH 5.0) was added and mixed. To this mixture 0.6 ml of 2.5% glutaraldehyde was added dropwise with stirring at room temperature. It was allowed to stand at room temperature for 3 hours, homogenized in 40.0 ml of 0.2 M PBS (pH 7.4), centrifuged at 650 g for 20 minutes. Supernatant was discarded. Homogenization and centrifugation were repeated three times. Equal volumes of the precipitate and strain specific antitoxoplasma serum were mixed, allowed to stand for one hour at 37°C and overnight at 4°C and then centrifuged at 650 g for 30 minutes. The supernatant was collected and used in the experiments.

Immunodiffusion - Double diffusion in gel technique of Ouchterlony (1949)

Precleaned microscope slides were coated with 0.3% agarose in distilled water. Agarose gel for immunodiffusion was prepared by dissolving 0.9 gm agarose in 100 ml 0.04 M Tris-buffered saline, pH 7.6 (see Appendix) in a boiling water bath. When dissolved, 1 ml of 0.1 M sodium azide was added (as preservative) and poured onto the precoated slides on a horizontal level surface in 3.5 ml volumes per slide. The agarose was allowed to gel for 10 minutes and the slides were stored in a moist, covered container in the refrigerator for at least 4 to 5 hours, and for not more than one week, before cutting wells.

A pattern consisting of a central well and 6 peripheral wells, equidistant from the central well, was cut in the gel using a template. The wells were opened by removal of the gel plug with a needle and gentle aspiration.

The wells were filled with appropriate samples and the precipitin bands were allowed to develop at room temperatures (21-23°C) in a moist covered container for 24 to 48 hours. The slides were then washed with borate saline, pH 8.0 (see appendix) for at least 24 hours with one change of the saline, to remove the excess of an unreacted proteins. The slides were then covered with moist filter paper strips and dried at room temperature. The filter paper strips were peeled off and the slides were stained with a solution of amido black (see Appendix) for 15 minutes. Excess stain was removed by washing the slides with repeated changes of a 10% solution of acetic acid followed by distilled water. The slides were dried by gently pressing over a blotting paper and stored for future reference.

Immunoelectrophoresis*

Agarose slides were prepared for IEP following the same procedure as for immunodiffusion except that barbital-acetate buffer, pH 8.6 (see Appendix) was used to dissolve agarose instead of Tris-buffered saline. Patterns consisting of longitudinal troughs and wells were cut in the gel with a template. The wells were opened by removal of the gel plug with a needle and gentle aspiration. Samples were inserted into the wells by capillary tubes. Agarose was taken in either 3 ml or 4 ml amounts per slide and electrophoresis was carried out with a DC voltage of 6 V/cm for either 60 minutes or 75 minutes as given under each experiment.

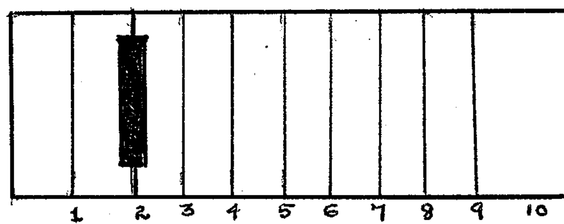
After the electrophoretic run, the gel in each pre-cut longitudinal trough was removed with a gel cutter. The trough(s) were filled with the appropriate antiserum(s), and the precipitin arcs were allowed to develop by allowing the slides to stand in a moist, covered container

* Universal Electrophoresis Cell, Buchler Instruments

for 48 hours.

Washing and staining of the slides were similar to those in immunodiffusion.

When ten different fractions of each toxoplasma antigen were to be collected for immunodiffusion study following electrophoresis, agarose was poured in 4 ml amounts per slide. After allowing to gel, a transverse trough was cut keeping a paper template underneath the slides. After the electrophoretic run for 75 minutes, ten fractions were cut using a paper template (as shown below) underneath the agarose slides.



Each fraction was transferred to a test tube and labeled strainwise and fractionwise, kept in the refrigerator at -20^{0}C for 24 hours, and the antigenic material was eluted from the fractions by freezing and thawing two times.

<u>Indirect micro-radioimmunoassay for detecting antibodies directed to toxoplasma (RH) tissue culture antigen</u>:

For the purpose of dilution of antigen, serum, and antisera, PBS (pH 7.2) containing 0.5% bovine serum albumin (BSA) was used in the experiment. Toxoplasma (RH) antigen prepared from tissue culture was diluted 1-20 with the diluent and 0.1 ml of this antigen was added into each of 16 wells in a microtiter plate* and allowed to adsorb by incubating at 37^{0}C for two hours, after which the wells were washed with PBS (pH 7.2) five times. Each of the following was added in 0.1 ml amounts per well in a set of four wells, that is, in quadruplicates. Rabbit antiRH

serum that was absorbed with NMS in the ratio of 1:2, diluted 1-10 with the diluent, normal rabbit serum diluted 1-10, rabbit antiRH serum diluted 1-10, and rabbit antimouse serum** diluted 1-10. The plate was incubated at 37°C for one hour and the wells were gently washed five times with PBS. Then 0.1 ml ^{125}I-labeled sheep antirabbit IgG+ (1-200) was added into each of the 16 wells, incubated for 2 hours at room temperature, followed by washing five times with PBS. The plate was allowed to air dry and the wells were separated with a scissors and the radioactivity of individual wells was counted in a Packard Auto-Gamma Spectrometer for 2 minutes.

* Disposable Microtiter Plates, Cook Engineering Co., Alexandria, VA. and Sam Mateo, CA.

** Rabbit antimouse serum was kindly supplied by Dr. S. Fujimoto, Immunology Department, Medical College, Manitoba

\+ ^{125}I-labeled sheep antirabbit IgG was kindly supplied by Dr. I. Berczi, Immunology Department, Medical College, Manitoba.

Statistical analysis of results of neutralization

The null hypothesis, that the antisera had no effect and that two samples (Control vs Rest) from the same population were being observed, was tested with a chi-square test (assuming that the plate counts of plaques follow a Poisson distribution). This hypothesis was rejected with $p < 0.05$.

While comparing the differences among the five antisera (antiRH vs antiV16 vs antiC56 vs antiC37 vs antiBeverley), the null hypothesis that the different antisera were no different in their effect and that five samples from the same population were observed, was tested with a chi-square test. This hypothesis was rejected with $p < 0.05$.

In comparing two antisera at a time (for example, antiRH vs antiV16), the null hypothesis in each case was that there was no difference in their effects which was tested with a chi-square test. The null hypothesis was rejected if significance was found at the 5% level of significance.

In examining the direct versus the dilution method the null hypothesis was that the ratios of counts when neutralized to counts for the control sample were the same in both the direct and the dilution methods, that is,

$$\left(\frac{\text{counts neutralized-direct}}{\text{counts control-direct}} \Big/ \frac{\text{counts neutralized-dilution}}{\text{counts control-dilution}} \right.$$

$$= \frac{C_{direct}}{C_{control-1}} \Big/ \frac{C_{dilution}}{C_{control-2}}$$

$$= \left. 1 \right)$$

This means that the statistic $T = \log C_{direct} - \log C_{control-1} - \log C_{dilution} + \log C_{control-2} = 0$.

This T was used in a test of the null hypothesis.

A direct estimation of the standard error of the neutralization ratio for each antigen, antiserum combination, for example, RH-anti-Beverley was computed as follows: Assuming a Poisson distribution, the ratio,

$$r = \frac{C_{RH:antiBeverley}}{C_{RH:control}}$$, approximately has the variance,

$$\sigma^2(r) = r^2 \left(\frac{\sigma^2 C_{RH:antiBeverley}}{\mu^2 C_{RH:antiBeverley}} + \frac{\sigma^2 C_{RH:control}}{\mu^2 C_{RH:control}} \right)$$

$$= r^2 \left(\frac{1}{C_{RH:antiBeverley}} + \frac{1}{C_{RH:control}} \right)$$

While comparing the neutralization patterns of two antigens by various antisera it was assumed that the counts for each antiserum and antigen combination followed a Poisson distribution and therefore the distribution of counts among the antisera within an antigen type, given the total counts for all the antiserum samples within the type would be a multinomial distribution, with

$$P_{ij} = \frac{\lambda_{ij}}{\sum_{i=1}^{5} \lambda_{ij}}$$

$i = 1,2,3,4,$ & 5 antisera

$j = 1$ & 2 antigens

The null hypothesis was that $P_{i1} = P_{i2}$ for all i or that the two antigens cannot be distinguished by their distribution of counts after neutralization by various antisera. The chi-square test for the homogeneity of two distributions was used and the null hypothesis was rejected with $p < 0.05$.

Statistical analysis of virulence tests

i) Probit transformation

Amplification of the response by cumulative method as applied in Reed & Muench (1938) does not result in improvement in precision, since precision can be improved by increasing the number of animals per group only. In addition, there is no ready means for calculating the standard error of the end point in this method. Measuring the probability of response on a transformed scale, the normal equivalent deviate, was proposed by Gaddum as early as 1933 (Finney, 1971). The introduction of "probits" that is, probability units, by Bliss (1938) has afforded a more precise method for the analysis of median lethal dose (LD 50) estimate than Reed and Muench method. Litchfield and Fertig (1941) have advanced a graphic procedure which is simple and reliable for deriving the standard error of the LD50 data. Miller and Tainter (1944) have extended this procedure employing a log-probit graph paper for a simple graphic estimation of the LD50 and its standard error with ease and without loss of time.

The extent to which the values of sample means (xs) are concentrated around the population mean (μ) is determined by the magnitude of the parameter σ. Various normally distributed populations can be differentiated by their σ values. Areas of the normal curve in terms of the variable coded to the form $(x-\mu)/\sigma$ are called the "normal deviates". Bliss (1938) added 5 to the normal deviate values in order to avoid the occurrence of minus signs appearing in the normal deviates.

Quantal (all-or-none) responses characteristically follow a sigmoid curve when per cent response is plotted against log dose (Harris, 1964). Frequency distribution of graded responses following a normal bell-shaped curve can be converted to a sigmoid curve by accumulating freq-

uencies at successive measurement intervals and the sigmoid curve thus obtained may be transformed to probability units and can be used graphically to judge whether the distribution of the observed values is in agreement with that expected for a normal distribution since a linear relationship exists between probits and variables in such a pattern (Reid, 1968).

In probit graph, then, the probability-units are just the relative deviations of normal deviates along the base of the normal bell-shaped curve that has been transferred to the ordinate scale (Bryan, 1958), and plotted on a probit paper as percentages of responses or on an ordinary graph paper as probits (Natrella, 1966). Percentage responses are converted to probits by reference to published tables (Fisher and Yates, 1948; Finney, 1952). LD50 estimate is obtained from the graph by finding the corresponding value to probit 5 (or 50%) on the abscissa. Similarly, the standard deviation can be readily obtained by finding the response value corresponding to one probit unit, that is, the increment between any two probit values.

When experimental data on the relation between dose and mortality are obtained, estimation of the parameters can be made either graphically or arithmetically (Finney, 1971).

In the present study LD50 values were derived following two steps. The first step involved estimating ED50 dilution* (fifty percent effective dose) in respect of each strain of toxoplasma employing Computer Program ST 36 (Rollwagen, 1973) for the analysis by probit method and to obtain

* A log transformation of the dilutions was used since the Chi square goodness of fit test resulted in smaller Chi square values with this transformation. The Chi square values indicated a good fit.

the statistics arithmetically. Dilutions giving 100% to 0% kill were used in this. From the values thus obtained for the expected probits a probit line was drawn on a probit graph paper with dilutions plotted on a log scale on the abscissa and the percentage as well as probits on the ordinate. The 95% confidence limits were drawn for the probit line from the upper and lower fiducial limits obtained in the program. Observed values of dilutions were then plotted on the graph against their probit or percent kill.

In the second step, estimation of number of plaques in the undiluted suspension of toxoplasma (K) was calculated using the following formula, deleting the dilutions which gave unreliable plaque counts as determined by the coefficient of variation.

$$\text{Estimated count of plaques in 0.5 ml of undiluted toxoplasma suspension} = K = \frac{\sum X}{\sum r.d}$$

X = the observed number of plaques in each petri dish,

r = the number of replications in respect of each dilution, and

d = the dilution factor.

The LD50 value of each strain was obtained by the product of the corresponding ED50 dilution and the K value.

ii) Rankit transformation

The term rankit ("Rank Unit") was introduced by Ipsen and Jerne (1944), who applied it to the analysis of graded response data. Rankit values range from -3 to +3, and are numerically identical to normal deviates. However, unlike both normal deviates and probits, rankits are derived as the average of "expected" values for the observed results

ranked in order of degree or magnitude of individual response among the group. Numerical value of rankit varies with the size of the sample.

The observed data were placed in order of rank based on time-to-death in respect of each dilution, and their equivalent rankit values were obtained from the published tables for the sample size of ten (Rohlf and Sokal, 1969). The rankit values obtained were plotted as the ordinate on ordinary graph paper against the corresponding time-to-death in log days on the abscissa. When the data were similarly plotted against the response time in days, a fairly straight line was obtained. However, when the response time was transformed into log units (metameters), it resulted in obtaining a better straight line. The mean log RT50* represented by the rankit value of zero, and the standard deviation, represented by the slope and equivalent to the increment of response on the abscissa in log units corresponding to one rankit on the ordinate could be obtained from the graph. Mean log RT50 for other dilutions were similarly obtained. From the values for various dilutions giving 100% to 0% death in mice, the mean log RT50 for that particular strain was calculated.

Probit transformation was not applied for the analysis of RT50 since it is not strictly applicable in graded assays comprising fewer than twentyfive animals per dose group (Harris, 1964).

* Computer Program ST 34 (University of Manitoba) was employed to obtain the statistics arithmetically.

RESULTS

NEUTRALIZATION EXPERIMENTS

Study on the basic aspects on *in vitro* neutralization of toxoplasma(RH) in Vero monolayers by plaque assay (Shettigara, 1971)had revealed that with rabbit antiRH serum, neutralization obtained after an hour of neutralization at 37°C was relatively greater in the presence of heat inactivated guinea pig complement than in the presence of uninactivated guinea pig complement when assayed both by the direct method without "dilution" and by the standard method with "dilution". In the absence of guine pig complement, there was no neutralization of toxoplasma observed after one hour's neutralization by rabbit antiserum in the dilution method, whereas on direct assay without dilution there was significant amount of neutralization, though lesser than that observed in the presence of uninactivated complement as well as in the presence of inactivated complement. Rabbit antiRH serum employed in the above studies had an IFAT titre of 1:128.

Since several authors had found that addition of antigamma globulin at various concentrations had an enhancing effect on neutralization of viruses and bacteriophages by their respective homologous antisera (Hampar *et al*., 1968; Makela, 1966; and Carter *et al*., 1968), it was interesting to determine if antigamma globulin would enhance neutralization of sensitized toxoplasma.

In earlier studies by Shettigara (1971) chicken antiRH serum (IFAT 1:64) had given 33.6 to 51.9 per cent neutralization of toxoplasma (RH) in one hour of neutralization by the dilution method. Addition of guinea pig complement was observed to have an inhibitory effect on neutralization of toxoplasma by chicken antiserum unlike rabbit antiserum.

To investigate the effect of antichicken gamma globuline(ACGG) on neutralization by chicken antitoxoplasma serum the following experiment was carried out.

Experiment 1.

Effect of antichicken gamma globulin* on neutralization of toxoplasma(RH) by chicken antiRH serum.

In each of four control tubes A and four test system tubes A, 0.75 ml toxoplasma(RH) suspension in PBS-A with 2% calf serum plus PSF, at a concentration of about 4×10^6/ml was taken, and in each of four control tubes B, 0.75 ml chicken preimmune serum was taken. Similarly 0.75 ml chicken antiRH serum (IFAT 1:128) was measured into each of four test system tubes B. One set of A and B control tubes were kept in a water bath at 37°C separately, followed one minute later by a set of test system tubes A and B. It was followed by the second set of control and then the corresponding test system with an interval of a minute. When all the four sets of tubes had been placed in the water bath, that is, after 8 minutes, the contents of control A and B were mixed, and a minute later the contents of the test system A and B tubes and similarly the other tubes.

After 30 minutes of this mixing, to the first set of control and test system 0.75 ml of 1:10 ACGG diluted in PBS-A was added and to the second set of control and test system, 0.75 ml of 1:100 dilution of ACGG was added and mixed. To the other two sets of control and test systems 1:10 and 1:100 dilution of ACGG was added 60 minutes after their corresponding mixing times. Taking the time of addition of ACGG

* Rabbit ACGG (Sylvana) was kindly supplied by Dr. H.I. Sayed, Microbiology Department, Medical College, Winnipeg.

as zero neutralization time, 0.5 ml of the mixture was assayed for plaques at zero time, that is, immediately on addition of ACGG and mixing, and after allowing a neutralization time of 30 and 60 minutes, each time 0.5 ml of the final mixture being diluted in 100 ml of diluent (PBS-A with 2% calf serum plus PSF), that is, a 200-fold dilution and 0.5 ml of this diluted mixture was inoculated into each of the two Vero monolayers in 60 mm petri dishes.

Results of the experiment are presented in Table 2. When ACGG was added at a concentration of 1:10 (final concentration 1:30), there were hardly any plaques at 30 minutes and at 60 minutes' neutralization without regard to the time of addition of antigamma globulin whether after 30 or 60 minutes of mixing of toxoplasma and chicken antiserum. At 1:100 concentration (final concentration 1:300), the neutralization obtained was poor, indeed, considering the amount of neutralization obtained by chicken antiserum alone in earlier studies (Shettigara, 1971).

Experiment 2.

Effect of antirabbit gamma globulin* on neutralization of toxoplasma (RH) by rabbit antiRH serum and guinea pig complement.

Experiment was carried out as above, this time using rabbit antiRH serum and guinea pig complement in place of chicken antiRH serum, and antirabbit gamma globulin (ARGG) in place of ACGG. 1:10 and 1:100 dilutions of ARGG in PBS-A was added separately 30 minutes after the mixing of toxoplasma-rabbit antitoxoplasma serum and guinea pig complement and assayed at zero, 30-, 60-, and 90-minutes. Control and test systems comprised 0.5 ml Toxoplasma gondii (RH) suspension in PBS-A with

* Goat ARGG (Sylvana) was kindly supplied by Dr. H.I. Sayed, Microbiology Department, Medical College, Winnipeg.

2% calf serum plus PSF at a concentration of about 4×10^6/ml, 0.5 ml PBS-A buffer, 0.5 ml guinea pig complement (1-32 dilution to contain 4 C'H50) in tubes A, 0.5 ml rabbit preimmune serum in control B tubes and 0.5 ml rabbit antiRH serum in test B tubes. ARGG in 1-10 and 1-100 dilutions was added in 0.5 ml volumes 30 minutes after the mixing of contents of A and B tubes. 0.5 ml of the final mixture was assayed at zero-, 30-, 60-, and 90-minutes on replicate Vero monolayers following a 200-fold dilution in the diluent as before.

Results are given in Table 3. Neutralization was erratic and was of poor magnitude in the presence of ARGG by rabbit antiserum and guinea pig complement.

Since the results of neutralization by chicken antiRH serum on addition of ACGG was disappointing, further investigation into the effect of addition of ARGG on neutralization of toxoplasma sensitized by rabbit antitoxoplasma serum only was not undertaken.

Experiment 3.

Comparison of neutralization of Toxoplasma(RH) by rabbit antiRH serum in the presence of heat inactivated guinea pig complement and uninactivated guinea pig complement.

Although similar comparative studies had been carried out previously (Shettigara, 1971) using rabbit antiRH serum with an IFAT titre of 1:128 and allowing neutralization at 37°C for one hour, it was of interest to compare neutralization in the presence of heat inactivated and uninactivated guinea pig complement, extending the period

TABLE 2

EFFECT OF ANTICHICKEN GAMMA GLOBULIN ON NEUTRALIZATION OF TOXOPLASMA (RH) BY CHICKEN ANTIRH SERUM

Neutralization Time	1:10 anti γ-gl. added after 30 min.			1:100 anti γ-gl. added after 30 min.			1:10 anti γ-gl. added after 60 min.			1:100 anti γ-gl. added after 60 min.		
	C *	T **	%N +	C	T	%N	C	T	%N	C	T	%N
	94,106	115,99		151,149	163,152		174,157	100,110		153,138	153,145	
0 hour	100 ¢	107	-	150	157	-	165	105	36.36	145	149	-
				158,138	121,138		0,2	0,0		164,140	116,120	
30 min.	0,0 ¢	0,0		148	129	12.84	1	0		152	118	22.37
				149,151	113,116					127,113	105,102	
60 min.	0,0 ¢	0,0		150	115	23.33	0,0	0,0		120	103	14.17

* Control system comprised 0.75ml suspension of toxoplasma (RH) at a concentration of about 4×10^6/ml in PBS-A with 2% calf serum, 0.75ml chicken preimmune serum and 0.75ml antichicken gamma globulin of appropriate dilution.

**Test system contained 0.75ml chicken antiRH serum (IFAT 1:128) instead of chicken preimmune serum; otherwise same as control system.

\+ Percent neutralization.

¢ Average number of plaques in two replicates.

TABLE 3

EFFECT OF ANTIRABBIT GAMMA GLOBULIN ON NEUTRALIZATION OF TOXOPLASMA (RH) BY RABBIT ANTIRH SERUM AND COMPLEMENT

NEUTRALIZATION	1:10 antiγ-gl. added after 30 minutes			1:100 antiγ-gl. added after 30 minutes		
TIME	C*	T**	%N+	C	T	%N
	58,50	56,56		56,60	50,46	
0 hour	54¢	56	-	58	48	17.24
	44,58	61,61		43,55	59,57	
30 minutes	51¢	61	-	49	58	-
	41,47	32,41		48,44	34,36	
60 minutes	44¢	37	15.91	46	35	23.91
	30,26	34,34		39,29	19,32	
90 minutes	28¢	34	-	34	25	26.47

* Control system comprised 0.5ml suspension of toxoplasma (RH) at a concentration of about 4×10^6/ml in PBS-A with 2% calf serum, 0.5ml complement (1-32), 0.5ml rabbit preimmune serum, and 0.5ml anti-rabbit gamma globulin of appropriate dilution.

**Test system contained 0.5ml rabbit antiRH serum instead of rabbit preimmune serum; otherwise same as control system.

+ Percent neutralization.

¢ Average number of plaques in two replicates.

of neutralization to four hours and employing rabbit antiRH serum with a higher IFAT titre, namely 1:1024.

In the test system with heat inactivated complement, 0.5 ml toxoplasma (RH) suspension containing approximately 4×10^6/ml in Dulbecco's PBS pH 7.5, was taken in a sterile stoppered test tube A, to which 0.5 ml inactivated guinea pig complement (at 56°C for 30 minutes) corresponding to 4 C'H50 units (1-32 dilution of guinea pig serum originally estimated to contain 256 C'H50 units/ml) were added.

In a sterile stoppered test tube B, 0.5 ml rabbit antiRH serum with a known titre of 1:1024 (IFAT) was taken and the contents of the two test tubes were mixed at zero 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 tubes. In the control system 0.5 ml rabbit preinoculation serum, previously tested and found negative in IFAT was used in place of rabbit antiRH serum in tube B. Tube A contained the same as in tube A of the test system. Test and control systems were similarly prepared with uninactivated guinea pig complement. All the tubes were shaken to mix the contents at frequent intervals. At 4 hour 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 100 ml PBS-A with 2% calf serum, plus PSF in each, thus bringing about a dilution of 200-fold. After thoroughly mixing, the diluted samples in 0.5 ml volumes were inoculated onto each of two Vero monolayers for plaque assay.

The results of the experiment are shown in table 4. There was significantly greater neutralization in the presence of inactivated complement than in the presence of uninactivated complement, after 4 hours' neutralization ($p < 0.05$).

Experiment 4.

Determination of optimum pH and optimum time for neutralization of T. gondii(RH) by rabbit antiRH serum in the presence of inactivated complement.

a) HBSS (1X)

HBSS (IX) with varying pH was prepared as shown in table 5.

Toxoplasma (RH) from the mouse exudate was collected in a small aliquot of physiological saline, counted and distributed in equal amounts into each of the four sterile bottles containing the same amount of buffer with different pH, namely, pH 7.95, 7.7, 7.3 and 6.8. Further dilutions of toxoplasma suspension were made in the corresponding pH buffer to obtain a concentration of approximately 4×10^6/ml of toxoplasma. Each pH had a control and test system. Test system comprised 1.0 ml of toxoplasma suspension in HBSS of particular pH, 1.0 ml HBSS(1X)of the same pH, 1.0 ml inactivated complement corresponding to 8 C'H50 (1-32 dilution of guinea pig serum which contained 256 C'H50 units before dilution), and 1.0 ml of rabbit antiRH serum. Control system had 1.0 ml rabbit preimmune serum in place of rabbit antiRH serum; otherwise, same as the test system. Taking toxoplasma suspension, HBSS, and inactivated complement in tube A, one tube for

TABLE 4

COMPARISON OF NEUTRALIZATION OF TOXOPLASMA (RH) BY RABBIT ANTIRH SERUM IN THE PRESENCE OF HEAT INACTIVATED COMPLEMENT AND UNINACTIVATED COMPLEMENT

NEUTRALIZATION	INACTIVATED COMPLEMENT			UNINACTIVATED COMPLEMENT		
TIME	C *	T **	%N +	C	T	%N
	160,164	148,154		136,138	131,143	
0 hour	162 ¢	151		137	137	
	156,150	63,53		118 110	68 66	
4 hours	153¢	58	62.1%	114	67	41.2%

* Control system comprised 0.5ml suspension of toxoplasma(RH) at a concentration of about $4x10^6$/ml in Dulbecco's PBS pH 7.5, 0.5ml rabbit preimmune serum , 0.5ml PBS buffer, and 0.5ml inactivated complement(1-32).

**Test system contained 0.5ml rabbit antiRH serum instead of rabbit preimmune serum; otherwise same as control.

+ Percent neutralization.

¢ Average number of plaques in two replicates.

control and one for test system, and taking rabbit preimmune serum in tube B for control and rabbit antiRH serum in tube B for test system and keeping tubes A and B of control sustem in a water bath at 37°C, say, at time zero minutes, and tubes A and B of test system at one minute (for buffer pH 6.5) and similarly at one minute intervals the control and test systems of the next pH as shown in table 6 and allowing 8 minutes for equilibration to 37°C, the contents of A and B tubes of control were mixed at minute 8, which was zero hour and 0.5 ml of the mixture was blown into 100 ml of diluent in a bottle. In like manner, one minute later, that is, at minute 9, the contents of the corresponding test system tubes A and B were mixed and 0.5 ml of the mixture diluted in a separate bottle containing 100 ml of diluent. 0.5 ml of the 200-fold diluted mixture was inoculated into each of the two Vero monolayers and assayed for plaques. At 1 hour, 2 hours and 4 hours intervals 0.5 ml of the neutralization mixture from control and test systems was similarly diluted in separate bottles containing the diluent and assayed as above. At the end of four hours' neutralization, the pH of the contents of the control and test tubes was determined.

The results of neutralization and the change in pH after 4 hours' neutralization are given in table 7. Neutralization is seen increasing in magnitude with time. Maximum neutralization was seen in HBSS buffer pH 7.7 both at 2 hours and 4 hours of neutralization. Neutralization in HBSS pH 7.95 was nearly of the same proportion as in HBSS pH 7.7, namely 79.04% as against 82.05% in pH 7.7 ($p > 0.5$). Similarly, neutralization obtained in HBSS pH 7.3 and pH 6.8 when compared to that obtained in pH 7.7 were not significantly different ($0.3 < p < 0.4$, and

$0.1 < p < 0.2$ respectively). It can be further seen that at the end of 4 hours, the pH in all the tubes, irrespective of the original pH of HBSS buffer, had uniformly reached pH 8.6 to 8.8.

When the relative infectivity, that is, the logarithm of the proportion of the initial viable toxoplasma which remained unneutralized, was plotted as a function of time of neutralization, a straight line was not evident in all the four different pH systems, indicating that neutralization of toxoplasma did not follow first order of kinetics in this system (see figure 1).

TABLE 5

TABLE SHOWING THE PREPARATION OF HBSS (1X)

WITH DIFFERING pH

SOLUTIONS		Bottles to contain in ml								
		1	2	3	4	5	6	7	8	9
Solution A Na Cl 64 gm KCl 3.2 gm $MgSO_4$ 1.6 gm Glucose 8.0 gm.	in 400 ml D.W.	5	5	5	5	5	5	5	5	5
Solution B $CaCl_2$ 1.12 gm	in 800 ml D.W.	10	10	10	10	10	10	10	10	10
Solution C $NaHCO_3$ 2.8 gm	in 100 ml D.W.	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Solution D Na_2HPO_4 0.4 gm	in 200 ml D.W.	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5
Solution E KH_2PO_4 0.48 gm	in 200 ml D.W.	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5
D.W. QS		100	100	100	100	100	100	100	100	100
pH		7.95	7.7	7.55	7.4	7.3	7.2	7.1	7.0	6.8

TABLE 6

TIMING FOR VARIOUS PROCEDURES IN NEUTRALIZATION EXPERIMENT

TO DETERMINE pH AND OPTIMUM TIME

Procedure	pH 6.8		pH 7.3		pH 7.7		pH 7.95	
	C	T	C	T	C	T	C	T
	TIME IN MINUTES							
Keept A & B tubes separately in water both at time	0	1	2	3	4	5	6	7
Mix A & B and take 0.5 ml for dilution at 0 hour ie,	8	9	10	11	12	13	14	15
Take 0.5 ml for dilution at 1 hour, ie,	68	69	70	71	72	73	74	75
Take 0.5 ml for dilution at 2 hours, ie,	128	129	130	131	132	133	134	135
Take 0.5 ml for dilution at 4 hours, ie,	248	249	250	251	252	253	254	255

TABLE 7

DETERMINATION OF OPTIMUM pH AND TIME FOR NEUTRALIZATION OF TOXOPLASMA (RH) BY RABBIT ANTIRH SERUM IN THE PRESENCE OF INACTIVATED COMPLEMENT USING HBSS (1X)

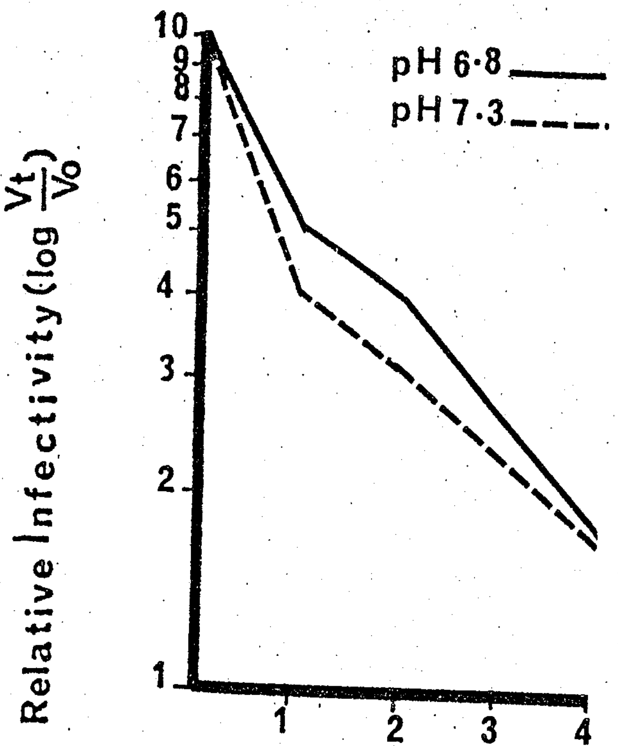
NEUTRALIZATION	pH 6.8			pH 7.3			pH 7.7			pH 7.95		
TIME	C *	T **	%N +	C	T	%N	C	T	%N	C	T	%N
	100	109		112	110		112	107		90	101	
	102	114		108	110		103	108		108	89	
0 hour	101¢	111	-	110	110	-	108	108	-	99	95	-
	96	55		99	46		111	40		103	39	
	110	62		111	42		93	43		113	34	
1 hour	103¢	58	43.7	105	44	58.1	102	42	58.8	108	37	65.7
	101	37		96	37		99	22		88	32	
	99	54		92	33		91	21		93	37	
2 hours	100¢	46	54.0	94	35	62.76	95	22	76.8	91	35	61.5
	60	22		72	17		79	14		64	10	
	70	17		74	21		78	14		60	15	
4 hours	65¢	20	69.2	73	19	73.97	78	14	82.05	62	13	79.04
pH after 4 hours	8.8	8.8		8.7	8.8		8.7	8.8		8.8	8.6	

* Control system comprised 1.0ml suspension of toxoplasma (RH) at a concentration of about $4x10^6$/ml in HBSS (1X) buffer of appropriate pH, 1.0ml inactivated complement (1-32), 1.0ml HBSS (1X) buffer of corresponding pH, and 1.0ml rabbit preimmune serum.

**Test system contained 1.0ml rabbit antiRH serum; otherwise same as control system.

+ Percent neutralization.

¢ Average number of plaques in two replicates.



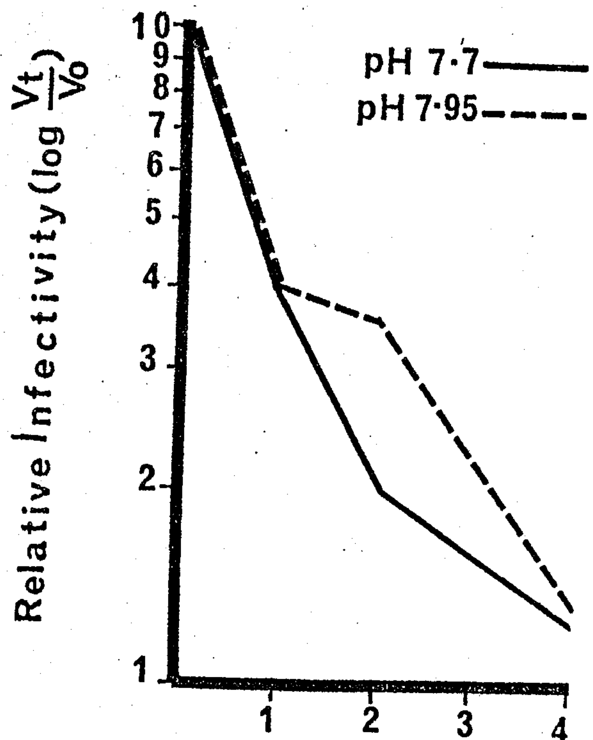


FIGURE 1.

KINETICS OF NEUTRALIATION OF TOXOPLASMA(RH) in HBSS OF FOUR DIFFERENT pH IN THE PRESENCE OF RABBIT SERUM AND INACTIVATED COMPLEMENT

b) HBSS (4X)

In the preceding experiment in which HBSS (IX) was used as buffer, the wide variation of pH at the end of 4 hours' neutralization from the original pH of the buffer indicated that the buffering capacity of HBSS (IX) was not sufficient to maintain the pH at the original level nor to keep the change within narrow limits. It was, therefore, decided to increase the concentration of Na_2HPO_4 and KH_2PO_4 of HBSS to 4X the original concentration and changing the individual amounts of these two components and keeping the total amount of the two constant as was done in the case of HBSS (IX) (Vide Table 5), HBSS (4X) with pH 6.8, 7.3, 7.7 and 7.95 were obtained and neutralization of T. gondii (RH) was carried out as in the above experiment. The results are furnished in table 8. With HBSS (4X), although maximum neutralization was seen at pH 7.95 at 4 hours' neutralization, it was not significantly different from those observed at pH 7.7 and 6.8. The pH at the end of 4 hours ranged from 8.0 to 8.4 as compared to 8.6 to 8.8 in the previous experiment with HBSS (IX). It is, however, important to note that the percentage of maximum neutralization obtained in HBSS (4X) is about 50% of that obtained in HBSS (IX). This significant difference in the proportion of neutralization of toxoplasma may be due to the change in osmolarity of HBSS (4X) from HBSS (IX), namely, 0.4618 and 0.433 respectively.

c) HBSS (20X)

The concentration of Na_2HPO_4 and KH_2PO_4 was increased 20-fold to that of their concentration in HBSS (IX) and the experiment was re-

TABLE 8

DETERMINATION OF OPTIMUM pH AND TIME FOR NEUTRALIZATION OF TOXOPLASMA(RH) BY RABBIT ANTIRH SERUM IN THE PRESENCE OF INACTIVATED COMPLEMENT USING HBSS (4X)

NEUTRALIZATION	pH 6.8			pH 7.3			pH 7.7			pH 7.95		
TIME	C*	T**	%N+	C	T	%N	C	T	%N	C	T	%N
	261 265	232 230		192 192	195 197		242 252	246 243		254 240	238 248	
0 hour	263¢	231		192	196		247	245		247	243	
	248 249	205 182		184 183	163 180		226 220	208 206		198 196	160 138	
1 hour	248¢	193	22.18	184	171	7.07	223	207	7.17	197	149	24.3
	247 228	140 150		190 180	180 170		222 218	180 170		170 174	135 125	
2 hours	238¢	145	39.08	185	175	5.41	220	175	20.46	172	130	24.4
	230 234	129 130		178 184	132 152		216 216	120 124		170 173	93 98	
4 hours	232¢	130	43.97	181	142	21.5	216	122	43.52	172	96	44.19
pH after 4 hours	8.2	8.3		8.3	8.0		8.4	8.1		8.0	8.3	

* Control system comprised 1.0ml suspension of toxoplasma(RH) at a concentration of about $4x10^6$/ml in HBSS(4X) buffer of appropriate pH, 1.0ml inactivated complement(1-32), 1.0ml HBSS(4X) buffer of corresponding pH, and 1.0ml rabbit preimmune serum.

**Test system contained 1.0ml rabbit antiRH serum instead of preimmune serum; otherwise same as control system.

+ Percent neutralization.

¢ Average number of plaques in two replicates.

TABLE 9

DETERMINATION OF OPTIMUM pH FOR NEUTRALIZATION OF TOXOPLASMA (RH) BY RABBIT ANTIRH SERUM IN THE PRESENCE OF INACTIVATED COMPLEMENT USING HBSS (20X)

NEUTRALIZATION / TIME	pH 6.8			pH 7.3			pH 7.7			pH 7.95		
	C *	T **	%N +	C	T	%N	C	T	%N	C	T	%N
0 hour	1,0	0,0		0,0	1,1		0,3	1,0		0,1	0,2	
4 hours	0,0 0 ¢	0,0 0		0,0 0	0,0 0		0,0 0	0,0 0		0,0 0	0,0 0	
pH after 4 hours	7.3	7.4		7.7	7.7		7.75	7.8		7.75	7.9	

* Control system comprised 1.0ml suspension of toxoplasma (RH) at a concentration of about 4×10^6/ml in HBSS (20X) buffer of appropriate pH, 1.0ml inactivated complement (1-32), 1.0ml HBSS (20X) buffer of corresponding pH, and 1.0ml rabbit preimmune serum.

**Test system contained 1.0ml rabbit antiRH serum; otherwise same as control system.

+ Percent neutralization.

¢ Average number of plaques in two replicates.

peated using the buffer with pH 6.8, 7.3, 7.7 and 7.95. Assay was made at zero hour and 4 hours. As can be seen from the results in table 9 the buffer (HBSS [20X]) was not conducive to the survival of T. gondii (RH) as there were hardly any plaques on assay even at zero hour. The osmolarity of HBSS(20X) was 0.6154 which must have been critical to survival of toxoplasma. The change in pH at the end of 4 hours neutralization did not exceed more than 0.6 units in any tube and the tubes with buffer pH 7.7 and 7.95 remained almost unchanged.

Experiment 5.

Determination of optimum pH and time for neutralization of toxoplasma (RH) by chicken antiRH serum using HBSS(IX).

As chicken antiRH serum was available from what was left over from earlier studies, it was decided to apply it for neutralization study to determine the effect of pH and time on chicken antibody to toxoplasma since neutralization of toxoplasma by chicken antibody was not enhanced by the addition of guinea pig complement. Therefore, the control and test systems comprised 1.0 ml each of toxoplasma(RH) suspension at a concentration of about 4×10^6/ml in HBSS(IX) of appropriate pH, and 2.0 ml of HBSS (lX) and in addition, 1.0 ml chicken preimmune serum in the control and 1.0 ml chicken antiRH in the test system. Neutralization was carried out exactly the same way as in experiment 4. The results are given in table 10. As with rabbit antiRH serum in HBSS(IX) buffer, chicken antiRH serum also manifested maximum neutralization at pH 7.7. The pH at the end of neutralization changed differently in different tubes as shown in table 10. Neutralization of toxoplasma(RH) by chicken antiRH serum, unlike rabbit antiRH serum plus

inactivated complement, seem to follow first order of kinetics for the first 2 hours of neutralization in all the four different pH before tending to form a plateau (see figure 2). While the per cent of neutralization was greater at pH 7.7 than at pH 7.95, the rate or speed of neutralization was greater at pH 7.95 than at pH 7.7 (k values of 0.115 and 0.112 respectively) as judged by the slope of the curves in figure 2. First order kinetics indicated by the straight line would suggest that the amount of toxoplasma being neutralized per unit time is a constant proportion of the residual viable toxoplasma population at any moment during the first two hours of neutralization and that the toxoplasma population is nearly homogeneous in an immunological sense.

Experiment 6.

Determination of optimum pH for neutralization of toxoplasma(RH) by rabbit antiRH serum in the presence of inactivated complement using Dulbecco's PBS(IX).

Since Dulbecco's PBS has higher concentrations of Na_2HPO_4 and KH_2PO_4 than HBSS (see Appendix, F,G, H and I) and has no sodium bicarbonate in it unlike HBSS, it was of interest to determine a) the effect of this buffer at various pH on the proportion of neutralization of toxoplasma and b) the fluctuation of pH of this buffer for a comparison with that using HBSS(IX) buffer.

PBS(IX) buffer with different pH was prepared on similar lines as HBSS(IX) as outlined in table 11, changing the amount of individual phosphates without changing the ionic strength. Neutralization proced-

ure was similar to that of experiment 4, with the exception that assay for surviving plaques was made at zero hour and four hours only. The results shown in table 12 clearly demonstrate that maximum neutralization is attained at pH 7.65 as compared to those at pH 7.5, 7.8, 6.5 and 7.0. However, the difference in neutralization at pH 7.8, 7.5 and 6.5 was not sifnificant from that at pH 7.65 at the 5% level ($p < 0.3$, $p < 0.4$, and $0.10 < p < 0.20$ respectively). Maximum neutralization of 68.0% reached at pH 7.65 is, however, considerably lower than that reached when HBSS(IX) was used as the buffer. Final pH at the end of 4 hours' neutralization ranged between 7.8 and 7.9 in all the tubes as compared to 8.6 to 8.8 in HBSS(IX), indicating that Dulbecco's PBS has a better buffering capacity than HBSS. However, since the proportion of neutralization of toxoplasma was lower than in HBSS, it was decided to use HBSS(IX) buffer pH 7.7 in neutralization experiments to follow.

Experiment 7.

Determination of optimum amount of heat-inactivated complement for neutralization of T. gondii(RH) by rabbit antiRH serum.

Guinea pig serum known to contain 256 C'H50 units per ml was diluted 1-32, 1-28, 1-24 and 1-16 with cold physiological saline in separate sterile test tubes to contain 4, 5, 6 and 8 C'H50 units in 0.5 ml respectively, and the test tubes were kept in a water bath at 56°C for 30 minutes just prior to use in the experiment. In the control tubes 0.5 ml each of toxoplasma (RH) suspension at a concentration of about 8×10^{6}/ml, and 0.5 ml HBSS, pH 7.7, 0.5 ml inactivated

complement of appropriate dilution and 0.5 ml of rabbit preimmune serum was taken. In the test system, the only change involved addition of 0.5 ml of rabbit antiRH serum in place of 0.5 ml of rabbit preimmune serum of the control system. Neutralization was carried out at 37°C as stated earlier and assay for plaques were made at zero hour and at 4 hours, after a 200-fold dilution. The proportion of neutralization observed in the presence of 4, 5, 6 and 8 C'H50 units of inactivated complement as obtained by the difference from their respective controls are given in Table 13.

While the percentage of neutralization gradually increased with the increase in inactivated complement units, maximum neutralization being obtained at 8 C'H50 units of inactivated complement, the differences in neutralization at 6 and 8, or at 5 and 8 or at 4 and 8 C'H50 units are not significant. ($p > 0.5$,. $p > 0.4$, and $p > 0.4$ respectively).

It was observed that the proportion of neutralization on the whole was very low compared to the results obtained earlier in experiment 4. The concentration of toxoplasma at about 8 x 10^{6}/ml, taken with a view to obtain, for certain, sufficient number of plaques on the monolayers, lest the experiment become obsolete for want of sufficient plaque numbers at the end of 4 hours' neutralization, seemed to be too high and might have resulted in antigen-excess and hence proportionate reduction in neutralization. In experiments hereafter, when inactivated complement was used, guinea pig serum diluted 1-16 and inactivated at 56°C for 30 minutes prior to use in the experiment was employed.

TABLE 10

DETERMINATION OF OPTIMUM pH AND TIME FOR NEUTRALIZATION OF TOXOPLASMA (RH) BY CHICKEN ANTIRH SERUM

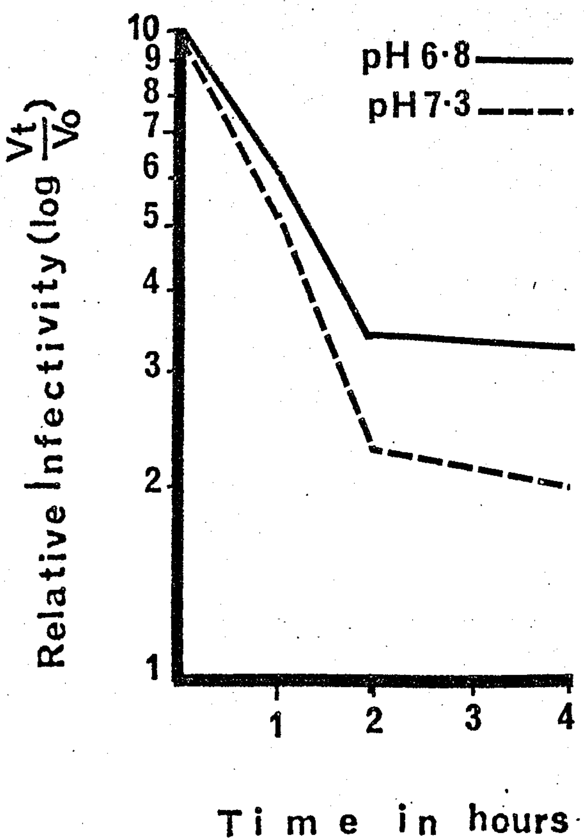
NEUTRALIZATION	pH 6.8			pH 7.3			pH 7.7			pH 7.95		
TIME	C *	T **	%N +	C	T	%N	C	T	%N	C	T	%N
	272	253		290	307		318	307		345	312	
	228	255		316	304		330	303		315	300	
0 hour	250 ¢	254	-	303	306	-	324	305	-	330	306	-
	199	165		274	154		276	168		216	117	
	194	145		238	168		290	158		205	136	
1 hour	196 ¢	155	20.9	256	161	37.1	283	163	42.4	210	126	40.0
	140	82		142	71		126	44		82	44	
	-	90		143	70		132	56		96	48	
2 hours	140 ¢	86	38.6	142	70	50.7	129	50	61.2	89	46	48.3
	142	75		132	63		106	33		66	35	
	136	93		144	64		121	40		82	28	
4 hours	139 ¢	84	39.6	138	64	53.6	113	36	68.1	74	32	56.8
pH after 4 hours	7.3	7.3		8.8	9.4		8.8	8.9		9.4	9.4	

* Control system comprised 1.0 ml suspension of toxoplasma (RH) at a concentration of about 4×10^6/ml in HBSS (1X) of appropriate pH, 2.0ml of the buffer of corresponding pH, and 1.0ml chicken preimmune serum.

**Test system contained 1.0ml chicken antiRH serum instead of chicken preimmune serum; otherwise same as control system.

+ Percent neutralization.

¢ Average number of plaques in two replicates.



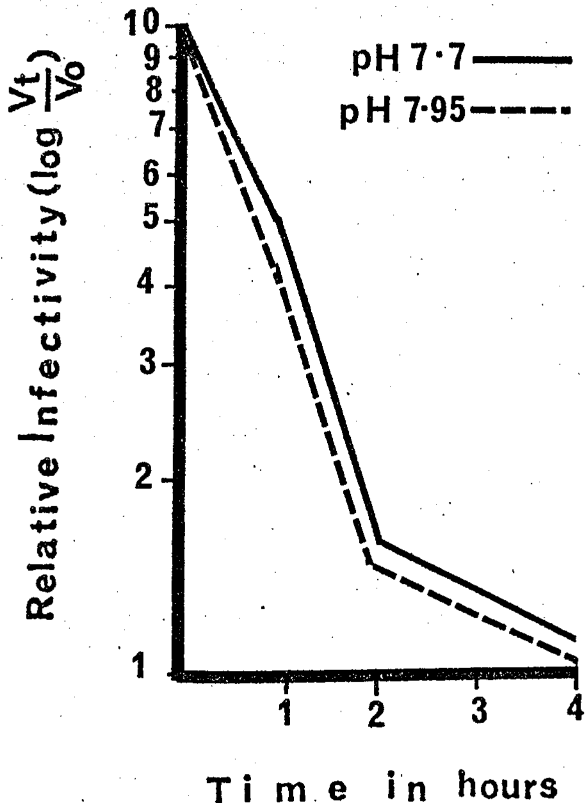


FIGURE 2.

CURVES SHOWING THE KINETICS OF NEUTRALIZATION OF TOXOPLASMA (RH) IN HBSS OF FOUR DIFFERENT pH IN THE PRESENCE OF CHICKEN ANTIRH SERUM

TABLE 11

Table showing the preparation of Dulbecco's PBS (IX) with differing hydrogen ion concentrations

Solutions	STERILE BOTTLES TO CONTAIN IN ML.								
	1	2	3	4	5	6	7	8	9
I. PBS-A (10X) Na Cl 40.0 gm k Cl 1.0 gm D.W. 500 ml	10	10	10	10	10	10	10	10	10
II. PBS-Na_2HPO_4 (20X) Na_2HPO_4 11.5 gm D.W. 500 ml	9	8	7	6	5	4	3	2	1
III PBS-KH_2PO_4 (20X) KH_2PO_4 2.0 gm. DW. 500 ml	1	2	3	4	5	6	7	8	9
IV. PBS-B (100X) $CaCl_2$ 5.0 gm. DW. 500 ml	1	1	1	1	1	1	1	1	1
V. PBS-C (100X) Mg Cl_2 $6H_2O$ 5.0 gm DW 500 ml.	1	1	1	1	1	1	1	1	1
VI. D.W.	78	78	78	78	78	78	78	78	78
pH	7.8	7.65	7.5	7.4	7.15	7.0	6.9	6.7	6.5

TABLE 12

DETERMINATION OF OPTIMUM pH FOR NEUTRALIZATION OF TOXOPLASMA (RH) BY RABBIT ANTIRH SERUM IN THE PRESENCE OF INACTIVATED COMPLEMENT USING DULBECCO'S PBS (1X)

TIME	pH 6.5			pH 7.0			pH 7.5			pH 7.65			pH 7.8		
	C*	T**	%N+	C	T	%N	C	T	%N	C	T	%N	C	T	%N
	71 67	70 30		130 152	150 152		156 160	144 148		156 142	136 140		126 143	128 136	
0 Hour	69¢	75	-	141	151	-	158	146	-	149	138	-	134	132	-
	46 56	23 24		173 178	90 94		150 156	63 53		148 152	46 50		124 140	51 52	
4 Hours	51¢	24	52.9	176	92	47.7	153	58	62.1	150	48	68.0	132	52	60.6
pH after 4 hrs.	7.8	7.85		7.85	7.9		7.8	7.85		7.85	7.9		7.9	7.9	

* Control system comprised 1.0ml suspension of toxoplasma (RH) at a concentration of about $4x10^6$/ml in Dulbecco's PBS buffer (1X) of appropriate pH, 1.0ml inactivated complement (1-32 dilution), 1.0ml Dulbecco's PBS buffer of corresponding pH, and 1.0ml rabbit preimmune serum.

**Test system contained 1.0ml rabbit antiRH serum instead of rabbit preimmune serum; otherwise same as control system.

\+ Percent neutralization.

¢ Average number of plaques in two replicates.

TABLE 13

DETERMINATION OF OPTIMUM AMOUNT OF HEAT-INACTIVATED COMPLEMENT FOR NEUTRALIZATION OF TOXOPLASMA (RH) BY RABBIT ANTIRH SERUM

TIME	4 C'H50			5 C'H50			6 C'H50			8 C'H50		
	C*	T**	%N+	C	T	%N	C	T	%N	C	T	%N
	403 387	401 398		426 363	348 362		381 394	379 417		344 313	327 345	
0 hours	395 ¢	399	-	394	355	-	387	398	-	328	336	-
	298 307	187 177		308 325	196 177		360 340	198 192		325 310	171 173	
4 hours	302 ¢	182	39.74%	317	187	41.01%	350	195	44.28%	318	172	45.91%

* Control system comprised 0.5 suspension of toxoplasma (RH) in HBSS buffer pH 7.7, 0.5ml inactivated complement of appropriate dilution, 0.5ml HBSS buffer, and 0.5mlrabbit preimmune serum.

**Test system contained 0.5ml rabbit antiRH serum instead of rabbit preimmune serum; otherwise same as control system.

+ Percent neutralization.

¢ Average number of plaques in two replicates.

Experiment 8

Comparison between neutralization at 4 hours and 8 hours of toxoplasma (RH) by antiRH serum applying direct and dilution methods.

Not being satisfied with the magnitude of neutralization of toxoplasma obtained at 4 hours' neutralization, it became necessary to investigate if extension of duration of neutralization would result in increased proportion of neutralization as compared to 4 hours. Both dilution and direct methods were employed side by side in respect of each of the two strains of toxoplasma, namely RH and C37 strains tested in separate experiments. In the standard method involving dilution, the control system comprised 0.5 ml each of toxoplasma of particular strain suspension in HBSS, pH 7.7, at an approximate concentration of 4×10^6/ml, HBSS buffer pH 7.7, inactivated complement 8 C'H50, and rabbit preinoculation serum. In the test system the only change involved 0.5 ml of corresponding strain specific rabbit antitoxoplasma serum in place of rabbit preimmune serum of the control system. 0.5 ml of the mixture, that is, toxoplasma-antiserum-inactivated complement, was assayed at zero, 4,and 8 hours on Vero monolayers after a 200-fold dilution in PBS with 2% calf serum, plus PSF, the diluent.

In the direct method 0.75 ml each of the toxoplasma suspension, buffer, inactivated complement and serum was taken and the concentration of toxoplasma was about 2×10^4/ml. At appropriate neutralization time 0.5 ml of the mixture was assayed directly on Vero monolayers.

For assay at zero time only one Vero monolayer was inoculated for each control and test system and replicate plates were used for assay

at 4 and 8 hours. The percentage of neutralization observed at 4 and 8 hours by both methods in respect of each strain of toxoplasma by its homologous antiserum is given separately in table 14 and 15.

With RH strain and antiRH serum-inactivated complement, there was a significant increase in neutralization at 8 hours as compared to 4 hours in the direct as well as in the dilution method. ($p < 0.001$ and $p < 0.05$ respectively.) Neutralization obtained in the direct method was slightly higher than the corresponding value in the dilution method both at 4 and 8 hours, though not significant ($0.2 < p < 0.30$, and $p > 0.4$ respectively) [vide table 14].

In the case of C37, neutralization by antiC37 serum in the direct method gave slightly higher values than in the dilution method at both 4 and 8 hours which were not significant at the 5% level of significance. Similarly, the small increase at 8 hours from 4 hours was not significant. On the other hand, there was a small decrease in per cent neutralization in the dilution method at 8 hours than at 4 hours but the difference was not significant at 5% level. This slight anomaly could be expected when the number of plaques per petri dish is small and can be influenced by the inherent experimental error (vide table 15). Small proportion of neutralization revealed at zero hour in the case of RH strain in both the methods might be due to the slight delay in assay after mixing the strain suspension and the antiserum-and-inactivated complement.

TABLE 14

COMPARISON BETWEEN NEUTRALIZATION AT 4 HOURS AND 8 HOURS OF TOXOPLASMA(RH) BY ANTIRH SERUM IN THE PRESENCE OF INACTIVATED COMPLEMENT

TIME	DILUTION			DIRECT		
	CONTROL*	TEST**	%N+	CONTROL#	TEST	% N
0 Hour	302ø	242	19.9%	306	232	24.2%
4 Hour	298	100		298	100	
	270	138		306	108	
	284@	119	59.86%	302	104	65.56%
8 Hours	66	15		122	16	
	60	9		128	18	
	63@	12	80.95%	125	17	86.40%

* Control system comprised 0.5 ml suspension of toxoplasma(RH) in HBSS pH 7.7, at a concentration of about $4x10^6$/ml, 0.5 ml HBSS buffer, 0.5 ml inactivated complement (1-16 dilution), and 0.5 ml rabbit preimmune serum.

** Test system contained 0.5 ml rabbit antiRH serum instead of rabbit preimmune serum; otherwise same as control system.

+ Percent neutralization.

ø Number of plaques in single plates.

@ Average number of plaques in two replicates.

In the direct method each of the components was taken in 0.75 ml amounts & the concentration of toxoplasma was 2×10^4/ml.

COMPARISON BETWEEN NEUTRALIZATION AT 4 HOURS AND 8 HOURS OF TOXOPLASMA(C37) BY ANTIC37 SERUM IN THE PRESENCE OF INACTIVATED COMPLEMENT

TIME	DILUTION			DIRECT		
	CONTROL*	TEST**	%N+	CONTROL #	TEST	%N
0 Hour	108ø	111	-	106	102	-
	77	48		75	47	
4 Hours	71	50		70	47	
	74@	49	33.78	72	47	34.72
	63	41		65	40	
8 Hours	57	44		57	38	
	60@	42	30.0	61	39	36.07

* Control system comprised 0.5 ml suspension of toxoplasma(C37) in HBSS pH 7.7 at a concentration of about 4×10^6/ml, 0.5 ml HBSS buffer, 0.5ml inactivated complement (1-16), and 0.5 ml rabbit pre-immune serum.

** Test system contained 0.5ml rabbit antiC37 serum instead of preimmune serum; otherwise same as control.

\+ Percent neutralization.

ø Number of plaques in single plates

@ Average number of plaques in two replicates.

\# In the direct method, each of the four components was taken in 0.75 ml amounts & the concentration of toxoplasma was 2×10^4/ml.

Experiment 9

Preliminary study on neutralization of two strains of toxoplasma by their homologous and heterologous antisera applying direct method.

Separate experiments were carried out for each strain of toxoplasma, namely, V16 and C56 strains.

Each strain of toxoplasma obtained from the peritoneal exudate of infected mice was collected in HBSS, pH 7.7 and diluted using the same buffer to obtain a concentration of approximately 2×10^4 toxoplasma/ml. 0.75 ml each of toxoplasma strain suspension, buffer, and inactivated complement (1-16 dilution) was taken in each of six sterile test tubes A. In one test tube B for control 0.75 ml rabbit preimmune serum was added. In other five test tubes B 0.75 ml each of different strain specific rabbit antiserum, namely antiRH, antiV16, antiC56, antiC37, and antiBeverley serum was taken. Keeping control A and B tubes separately in a water bath, followed in like manner by each strain specific antiserum B tubes and a corresponding A tube at an interval of a minute, thus allowing six minutes for each A and B tube for equilibration to 37°C, the contents of tube A and corresponding tube B were mixed and 0.5 ml of the mixture was assayed for plaques on a Vero monolayer plate at zero time and on replicate monolayers at 4 hours and 8 hours.

The results are given in figure 3.

Analysis of the results by Chi-square test revealed the following:

a) Neutralization of V16 strain:

Control Vs. Rest - highly significant ($p < 0.001$).

AntiRH Vs. antiV16 Vs. antiC56 Vs. antiC37 Vs. antiBeverley - highly

significant ($p < 0.001$).

(AntiC56 + antiC37) Vs (antiRH + antiV16 + antiBeverley) - highly significant ($p < 0.01$).

AntiRH Vs (antiV16 + antiBeverley) - significant ($p < 0.025$).

AntiC56 Vs antiC37 - not significant ($0.2 < p < 0.3$).

AntiC37 Vs antiBeverley - not significant ($0.10 < p < 0.20$).

b) Neutralization of C56 strain:

Control Vs Rest - not significant ($p > 0.1$).

AntiRH Vs antiV16 Vs antiC56 Vs antiC37 Vs antiBeverley - not significant ($p > 0.5$).

Experiment 10

Neutralization of the five strains of toxoplasma by their homologous and heterologous antisera employing both direct and dilution methods simultaneously.

While separate experiments were run for each strain of toxoplasma, both direct and dilution methods were carried out side by side using the same strain suspension at different concentrations in respect of each strain in the direct and dilution methods.

Direct method of neutralization in respect of each strain of toxoplasma was carried out the same way as in the preceding experiment excepting that each of the four components was taken in 0.5 ml amounts instead of 0.75 ml and assay was carried out at zero and 8 hours.

Dilution method was carried out as described earlier in experiment 4 involving a 200-fold dilution. Each of the four components was taken in 0.5 ml amounts and assayed at zero and 8 hours. The concentration of toxoplasma strain suspension was 200 times greater (about 4×10^6/ml)

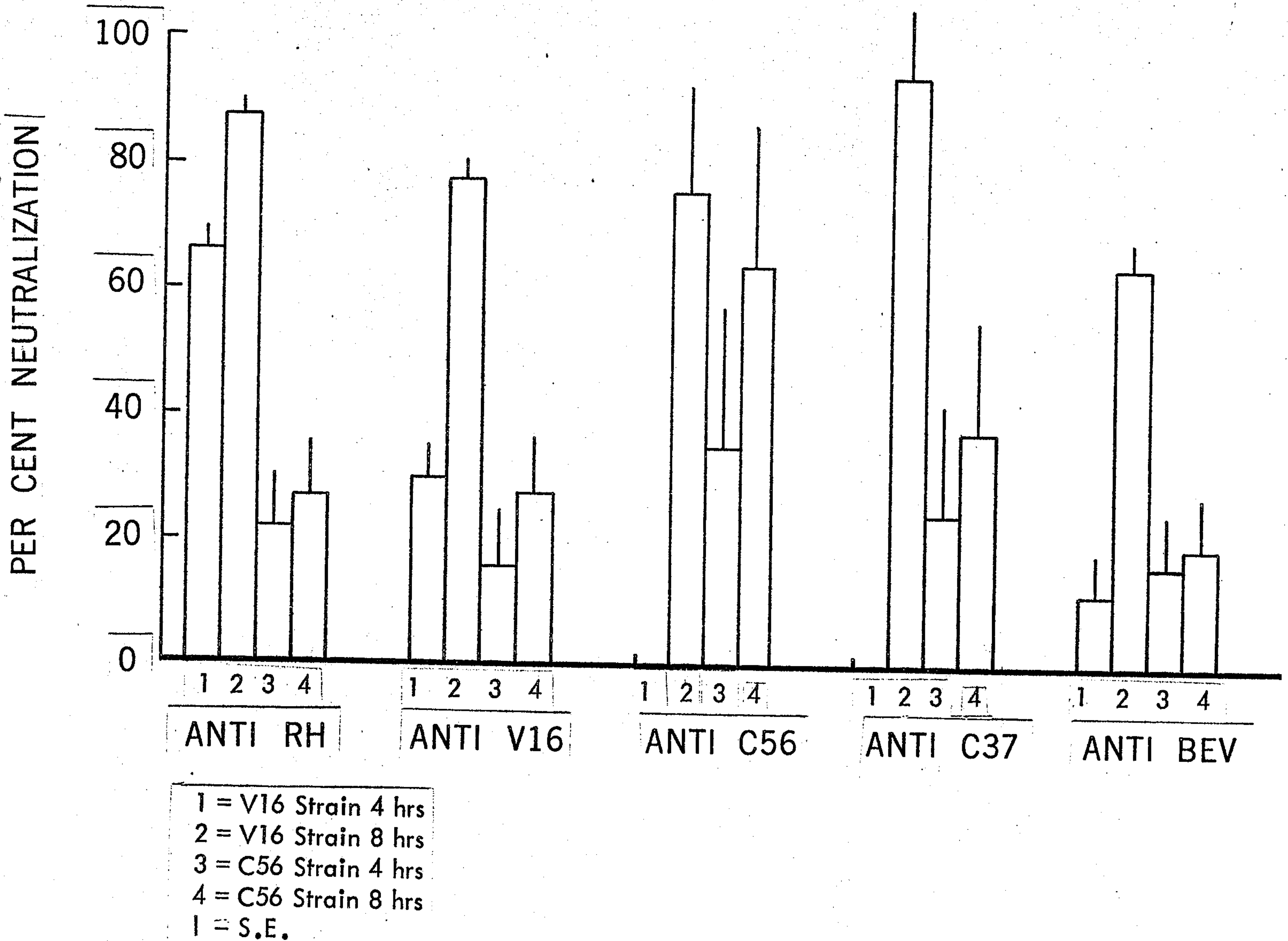


FIGURE 3.

Figure 3: Neutralization of V16 and C56 strains of Toxoplasma by the five strain-specific antisera at 4 and 8 hours in the direct method.

in the dilution method than in the direct method in which it was estimated at about 2 x 10^4/ml.

Results are given in figures 4A, 4B and 4C.

Though the proportion of neutralization varied somewhat when the results of neutralization in the direct and dilution methods were compared, the differences were not found significant at the five per cent level of significance.

Experiment 11

Comparison of Late and Early antiRH sera in neutralization of T.gondii(RH).

To obtain an idea of the relative merits of early and late antibodies in neutralization of toxoplasma, a comparative study of neutralization by early and late antiRH sera was carried out using RH strain of toxoplasma, bearing in mind, the observations of Strannegard (1967a) that 19S antibody type was more effective per molecule than 7S antibody in the immunoinactivation of toxoplasma.

In each of three test tubes A, 0.5 ml each of toxoplasma (RH) suspension at a concentration of about 4 x 10^6/ml, HBSS pH 7.7, inactivated complement (8C'H50) was taken. In the control tube B 0.5 ml of rabbit preimmune serum was added. In one of the test tube B 0.5 ml early antiRH serum was taken and in the other 0.5 ml of late antiRH serum was taken and neutralization was carried out as before for eight hours, 0.5 ml of the mixture being assayed after a 200-fold dilution on replicate Vero monolayers at zero and 8 hours.

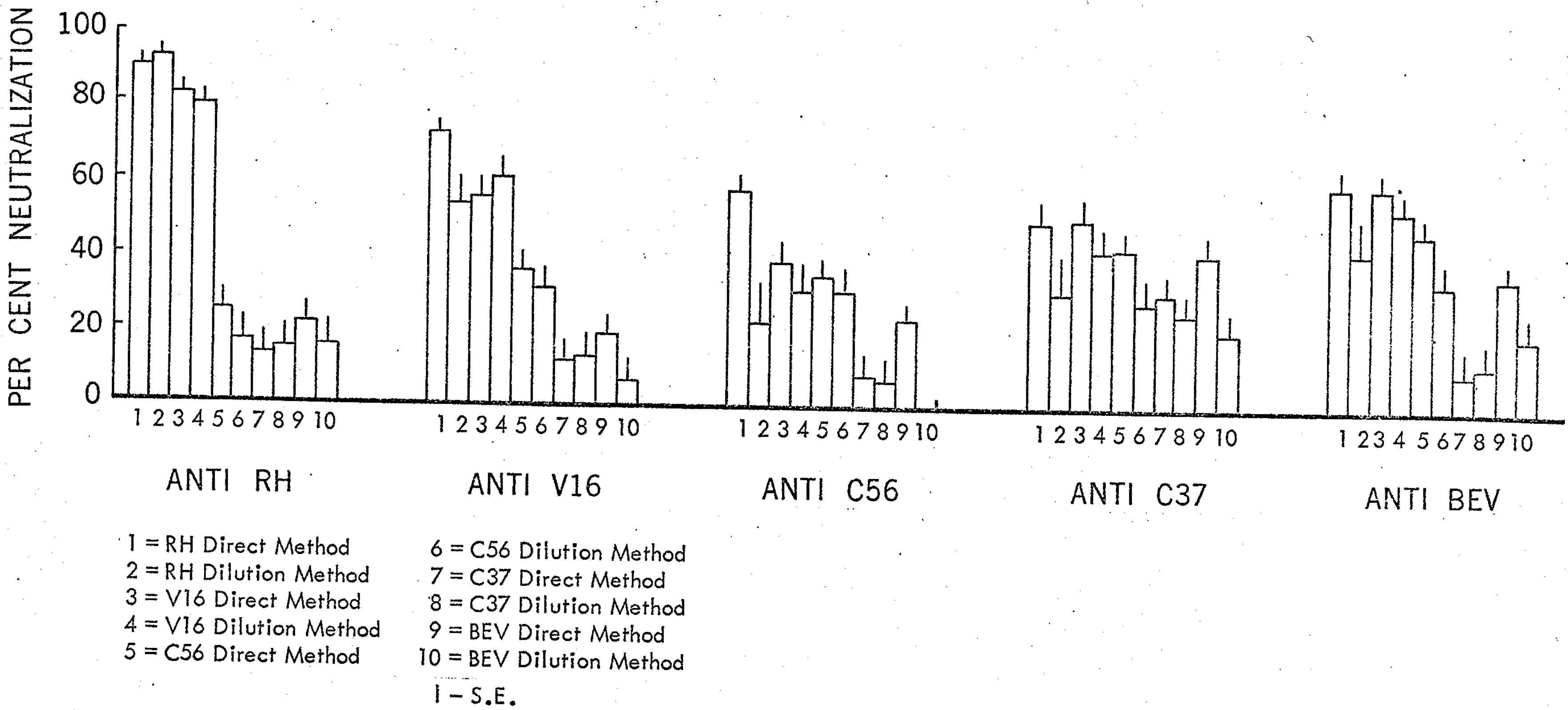


FIGURE 4A: Neutralization of five strains of Toxoplasma by each of the five strain-specific antisera by direct and by dilution methods.

Bar diagram showing neutralization antiserum-wise in direct & dilution methods.

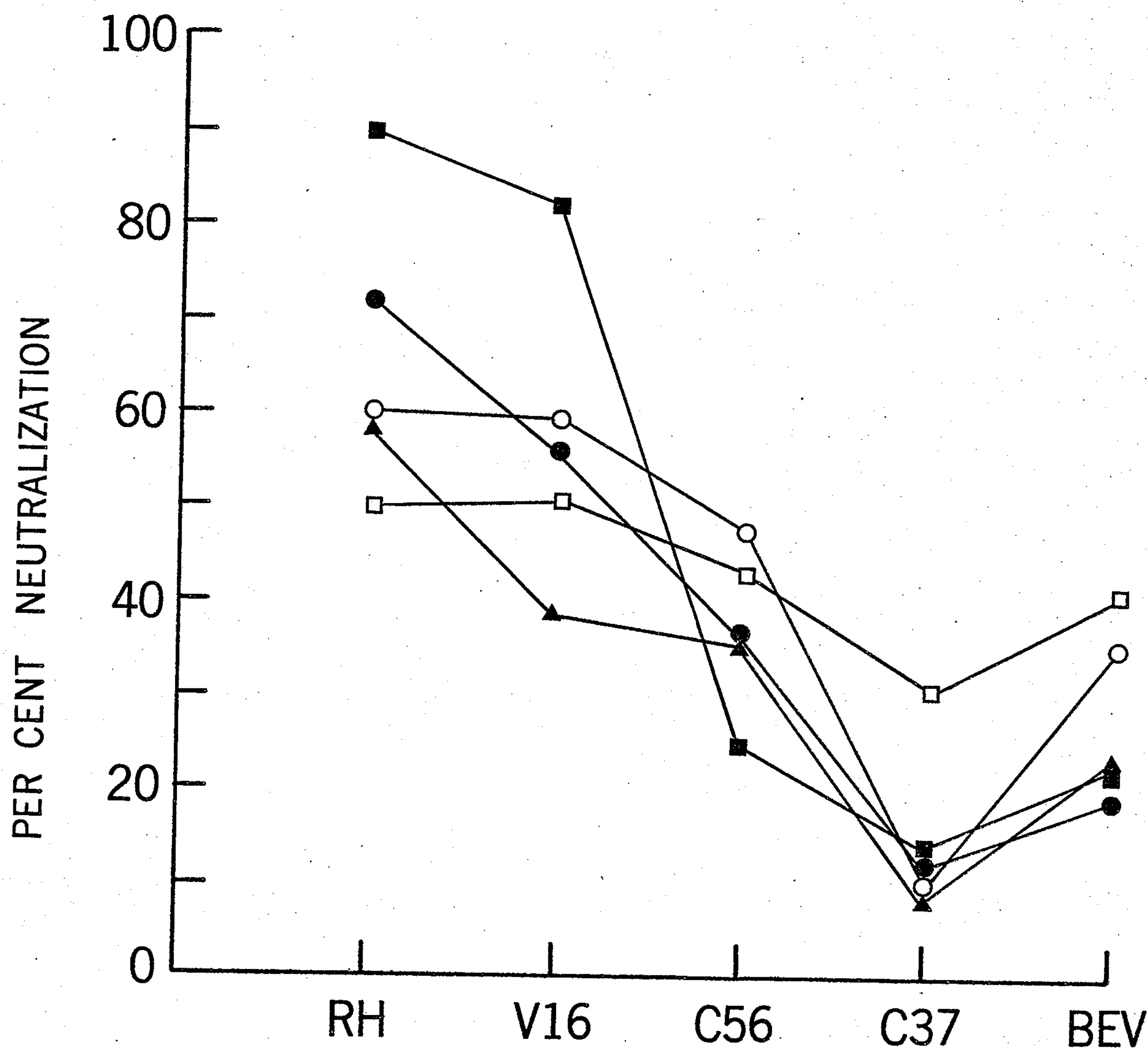


FIGURE 4B: Neutralization of five strains of Toxoplasma by each of the five strain-specific antisera by direct and by dilution methods.

Graph showing neutralization antigen-wise in direct method.

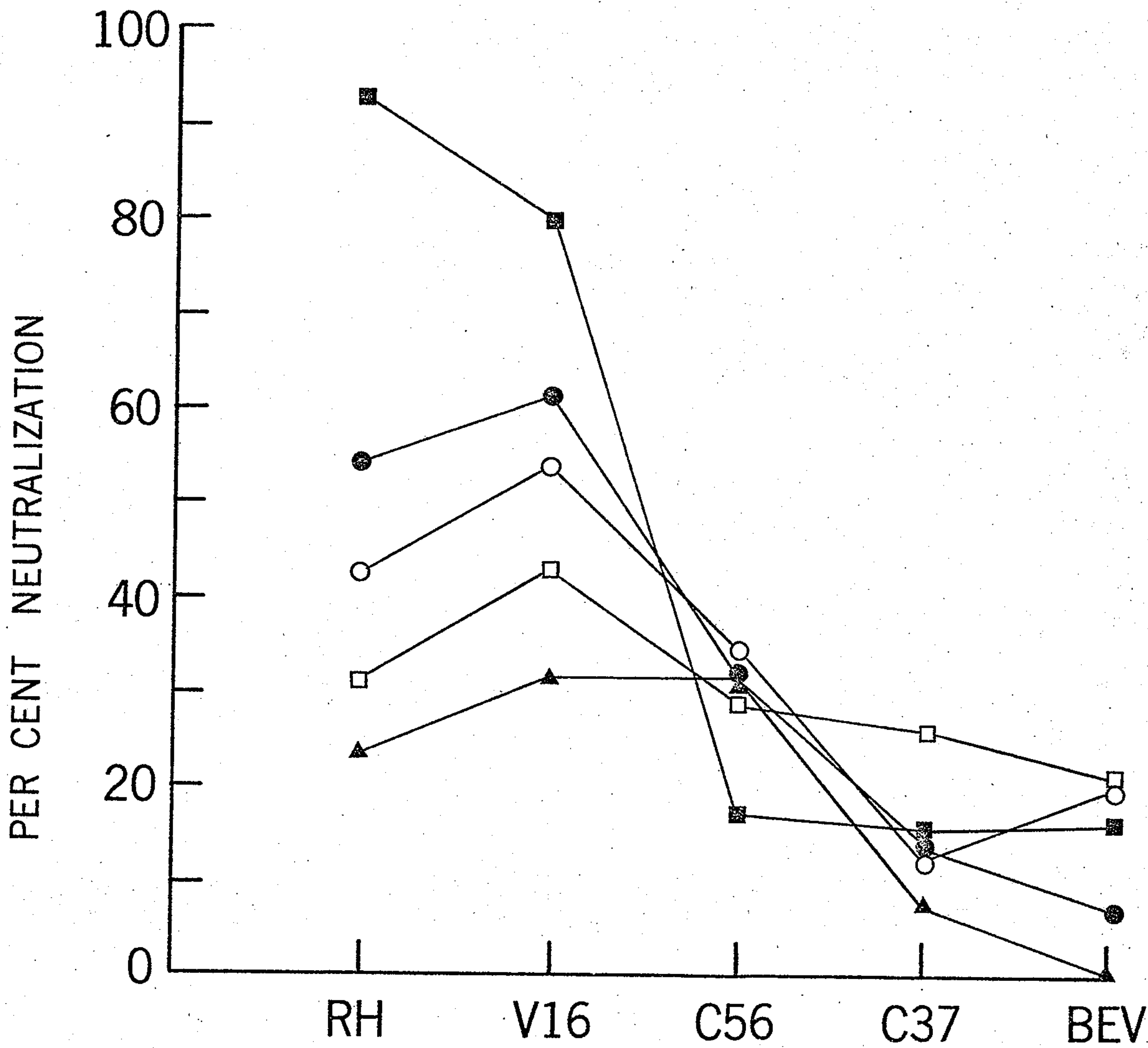


FIGURE 4C: Neutralization of five strains of Toxoplasma by each of the five strain-specific antisera by direct and by dilution methods.

Graph showing neutralization antigen-wise in dilution method.

Late antiRH serum with an IFAT titre of 1:1024 gave 71.3% neutralization compared to 19.62% obtained with early antiRH serum with an IFAT titre of 1:32 (Table 16).

Experiment 12

Study of the effect of rabbit antimouse serum on toxoplasma

Since toxoplasma obtained from the peritoneal exudate for neutralization experiments would be naturally contaminated with mouse protein(s) and the antitoxoplasma serum produced in rabbits against toxoplasma obtained similarly would contain antibodies, besides to toxoplasma, to mouse protein(s) (as confirmed by immunodiffusion studies) it was of interest to learn if the presence of antibodies to mouse protein(s) would influence the viability of toxoplasma for, if it did, it might reflect on the results of neutralization of toxoplasma by antitoxoplasma serum, thus obscuring the findings in neutralization assay.

Rabbit antimouse serum* was Millipore filtered, sterility tested and inactivated at 56°C for 30 minutes before being used in the experiment.

The control system comprised 0.5 ml each of toxoplasma(RH) suspension, HBSS buffer, pH 7.7, inactivated complement (8C'H50) in tube A and 0.5 ml rabbit preimmune serum in tube B. In the test system tube B contained 0.5 ml rabbit antimouse serum and the contents of test A tube was identical to that of the control A tube. Assay of surviving plaques was made after a 200-fold dilution at zero and 8 hours on replicate Vero monolayers.

It is clear from the results furnished in table 17, that rabbit antimouse serum, while it may interact with mouse protein(s) present in the toxoplasma suspension, does not in any way influence the viability or the plaque-forming ability of toxoplasma.

* Rabbit antimouse serum was obtained from Dr. Fujimoto, Department of Immunology.

TABLE 16.

COMPARISON OF LATE AND EARLY ANTIRH SERA IN NEUTRALIZATION OF TOXOPLASMA (RH)

TIME	CONTROL * (NRPIS)	LATE ANTI RH **	% N +	EARLY ANTI RH ¢	% N
0 Hour	375,385 380 @	385,379 382 @	-	385,396 391 @	-
8 Hours	207,211 209 @	62,58 60 @	71.3	170,166 168 @	19.62

* Control system comprised 0.5ml each of toxoplasma (RH) suspension at a concentration of about $4x10^6$/ml in HBSS nuffer pH 7.7, HBSS buffer, inactivated complement (1-16), and rabbit preimmune serum.

**Late antiRH test system contained 0.5ml late antiRH serum instead of rabbit preimmune serum; otherwise same as control system.

+ Percent neutralization.

¢ Early antiRH system contained 0.5ml early antiRH serum instead of rabbit preimmune serum; otherwise same as control system.

@ Average number of plaques in two replicates.

Experiment 13.

Effect of dilution of antiRH serum on neutralization of different strains of toxoplasma employing both direct and dilution methods.

While separate experiments were run for each strain of toxoplasma, both direct and dilution methods were carried out simultaneously in respect of individual strains of toxoplasma employing rabbit antiRH serum undiluted as well as diluted in sterile physiological saline. Neutralization procedures were similar to those in experiment 10.

The results are given in figure 5. While there was uniformly less neutralization by antiRH serum diluted 1-4 as compared to undiluted antiRH serum of homologous and heterologous strains in both direct and dilution methods, the difference in neutralization between undiluted and 1-4 diluted antiRH serum was not significant.

Experiment 14

Neutralization of the five strains of toxoplasma by their homologous and heterologous antisera (concentrated) employing dilution method; consistency of experiments.

From an appreciation of the results of the foregoing experiments on neutralization of toxoplasma and with a view to enhance the neutralization, all the strain specific antisera* and the preimmune serum were concentrated about three times, dialyzed against physiological saline and Millipore filtered as described in materials and methods for use in this and in the experiments to follow.

Dilution method of neutralization was carried out as described earlier in experiment 10, involving a 200-fold dilution. Each of four components

* IFAT titres of concentrated and unconcentrated antisera are given in table 18.

TABLE 17

EFFECT OF RABBIT ANTIMOUSE SERUM ON TOXOPLASMA (RH)

TIME	CONTROL *	TEST ** RABBIT ANTI MOUSE SERUM
0 Hour	375,385 380 +	380,390 385+
8 Hours	207,211 209+	209,216 213+

* Control system comprised 0.5ml each of toxoplasma suspension in HBSS buffer pH 7.7 at a concentration of about $4x10^6$/ml, HBSS buffer, inactivated complement and rabbit preimmune serum.

**Test system contained 0.5ml rabbit antimouse serum instead of rabbit preimmune serum; otherwise same as control system.

+ Average number of plaques in two replicates.

FIGURE 5.

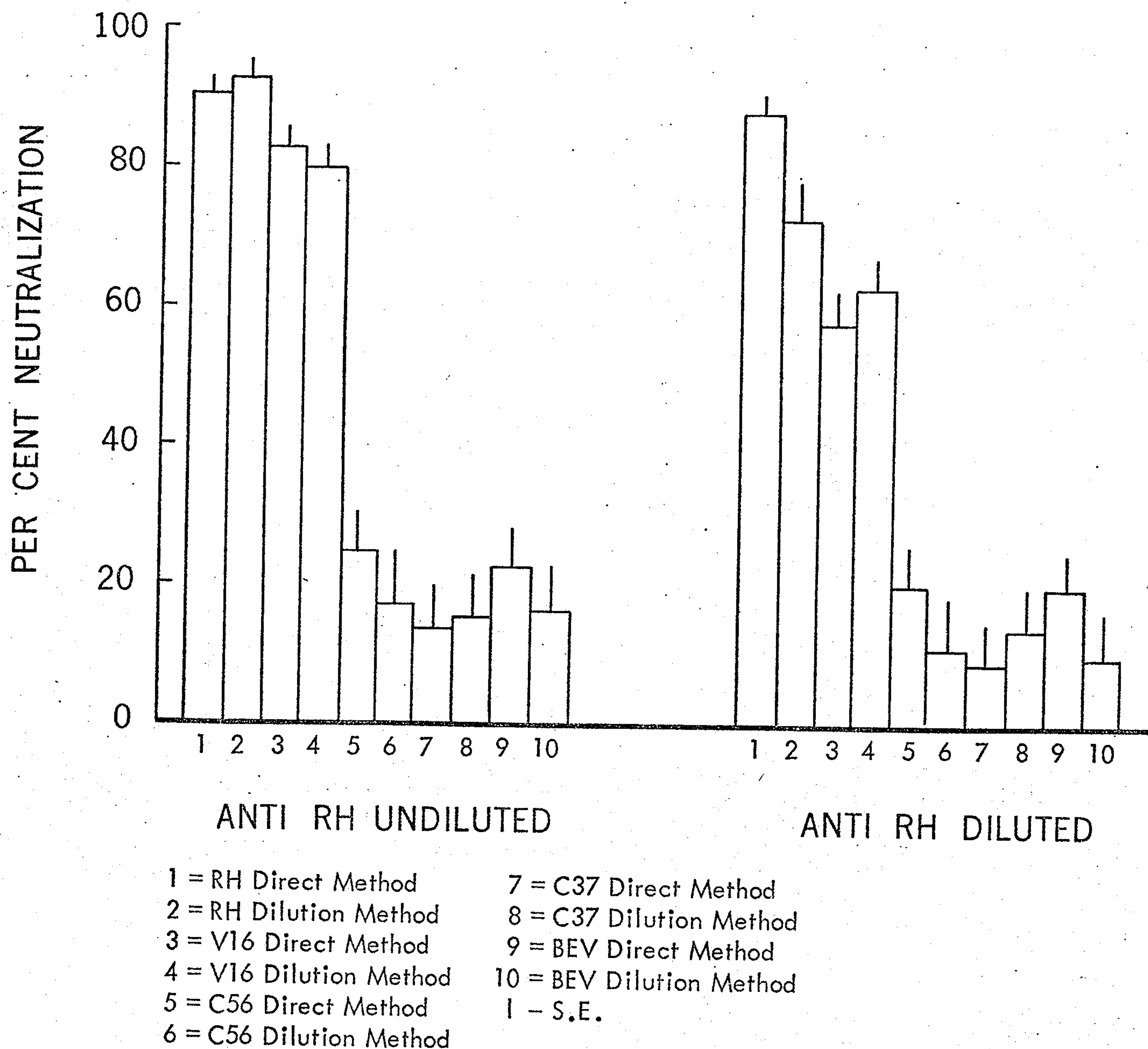


FIGURE 5: Effect of dilution of antiRH serum on neutralization of different strains of Toxoplasma in both direct and dilution methods.

of the reaction system, namely, toxoplasma strain suspension in HBSS pH 7.7 at an approximate concentration of 4×10^6/ml, HBSS pH 7.7, inactivated complement (guinea pig serum diluted 1-16), and serum was taken in 0.5 ml amounts and assayed at zero and 8 hours. 1.0 ml of the 200-fold diluted mixture was inoculated into replicate Vero monolayers instead of the usual 0.5 ml, in order to obtain greater number of plaques to facilitate better accuracy and lest the experiment should suffer for want of sufficient number of plaques through attrition for 8 hours at 37°C.

Separate experiments were carried out for each strain of toxoplasma and the results of two such experiments for each individual strain of toxoplasma are summarized in figures 6A, 6B and 6C.

The following variations of significance in the proportions of neutralization of toxoplasma were observed between two identical experiments in respect of each strain of toxoplasma carried out at different times by the dilution method.

With strain RH, neutralization by antiRH Vs antiV16 was highly significant in experiment 1 ($p < 0.001$) and significant in experiment 2 ($0.01 < p < 0.025$).

With strain V16, neutralization by antiRH Vs antiV16 was not sifnificant in experiment 1 ($0.1 < p < 0.2$) whereas it was highly significant in experiment 2 ($0.001 < p < 0.005$).

With strain Beverley, neutralization by antiC37 Vs antiBeverley was significant in experiment 1 ($0.025 < p < 0.05$) whereas in experiment 2 it was not significant ($p > 0.5$). On the other hand, neutralization by antiRH Vs antiV16 Vs antiC56 Vs antiC37 Vs antiBeverley was not significant in experiment 1 unlike in experiment 2 in which it was significant

($0.30 < p < 0.40$, $p < 0.05$ respectively).

Further, highly significant differences in neutralization between two experiments were observed in respect of neutralization of V16 by antiRH serum and of V16 by antiBeverley serum ($0.001 < p < 0.01$) and difference of significance was observed in neutralization of V16 by antiC37 serum ($0.01 < p < 0.05$).

Experiment 15

Neutralization with varying concentrations of toxoplasma, and constant antiserum-and-inactivated complement by dilution method.

The rather wide variations in the neutralization titre for any given strain of toxoplasma when the same experiment was repeated at a different time by dilution method was a quite disturbing feature observed. Dilution method was preferred over direct method for the obvious reason that the neutralization time allowed is exact as the 200-fold dilution at the appropriate neutralization time would prevent further neutralization by the 200-fold diluted antiserum whereas in the direct method the antiserum contained in the inoculation mixture can still act on toxoplasma on the inoculated indicator plates. However, since toxoplasma penetrate cells in tissue culture in a few seconds (Lunde et al, 1963) and over 95% of toxoplasma(RH) penetrated in 15 minutes after inoculation on Vero monolayers (Shettigara, 1971), the neutralization period allowed in the direct method would still carry meaning and for all practical purposes would be the same as in dilution methods, particularly when the neutralization time is extended as long as 8 hours. Counting of extracellular, live toxoplasma in haemocytometer under the microscope is known to be

TABLE 18

INDIRECT FLUORESCENT ANTIBODY TEST TITRES OF RABBIT ANTITOXOPLASMA SERA EMPLOYED IN NEUTRALIZATION TESTS.

ANTISERA	IFAT TITRE OF UNCONCENTRATED ANTISERA	IFAT TITRE OF CONCENTRATED ANTISERA
Anti RH	1:1024	1:512
Anti V16	1:1024	1:2048
Anti C56	1:256	1:128
Anti C37	1:256	1:128
Anti Beverley	1:256	1:128

FIGURE 6A

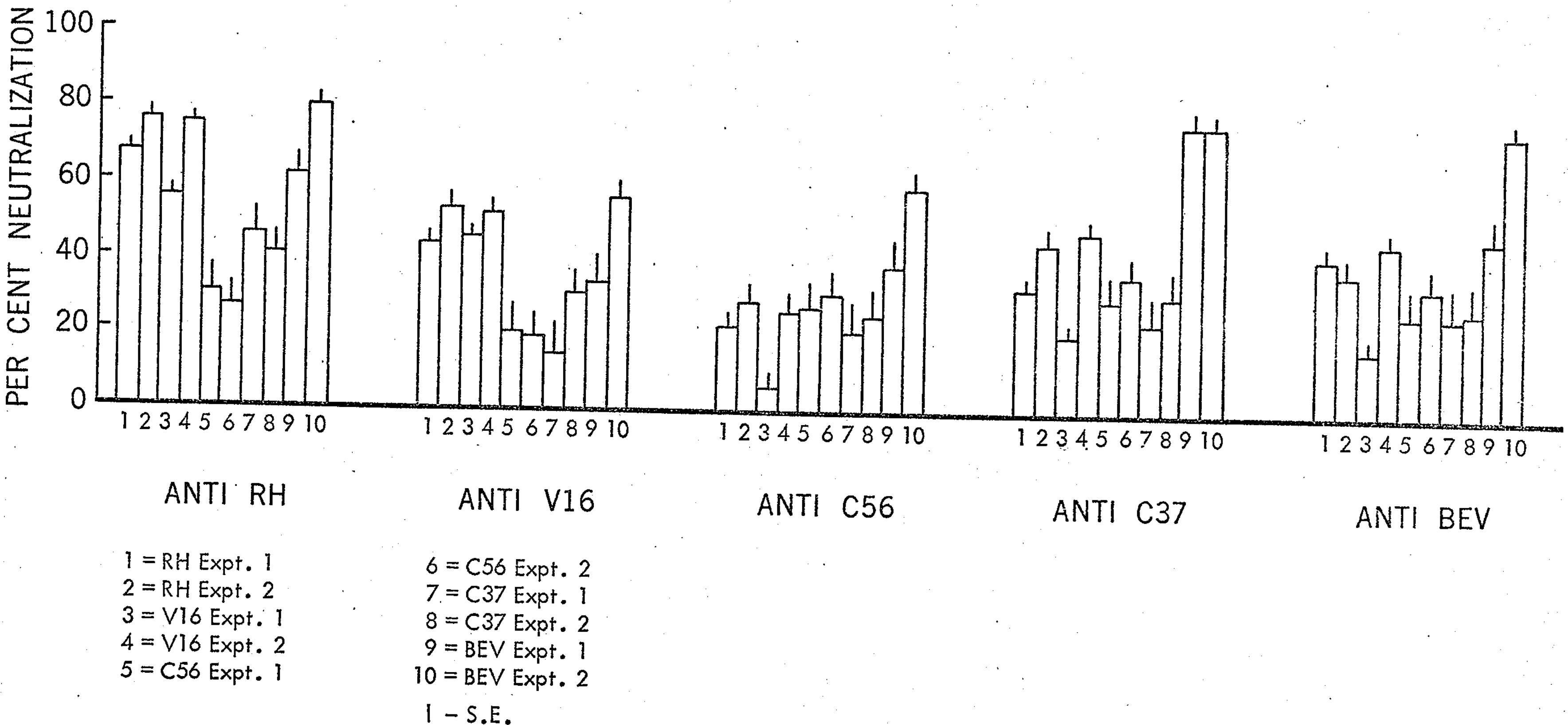


Figure 6. Neutralization of five strains of Toxoplasma by each of the five strain-specific antisera in the dilution method - results of two experiments.

Bar diagram showing neutralization antiserum-wise - 2 experiments.

FIGURE 6B

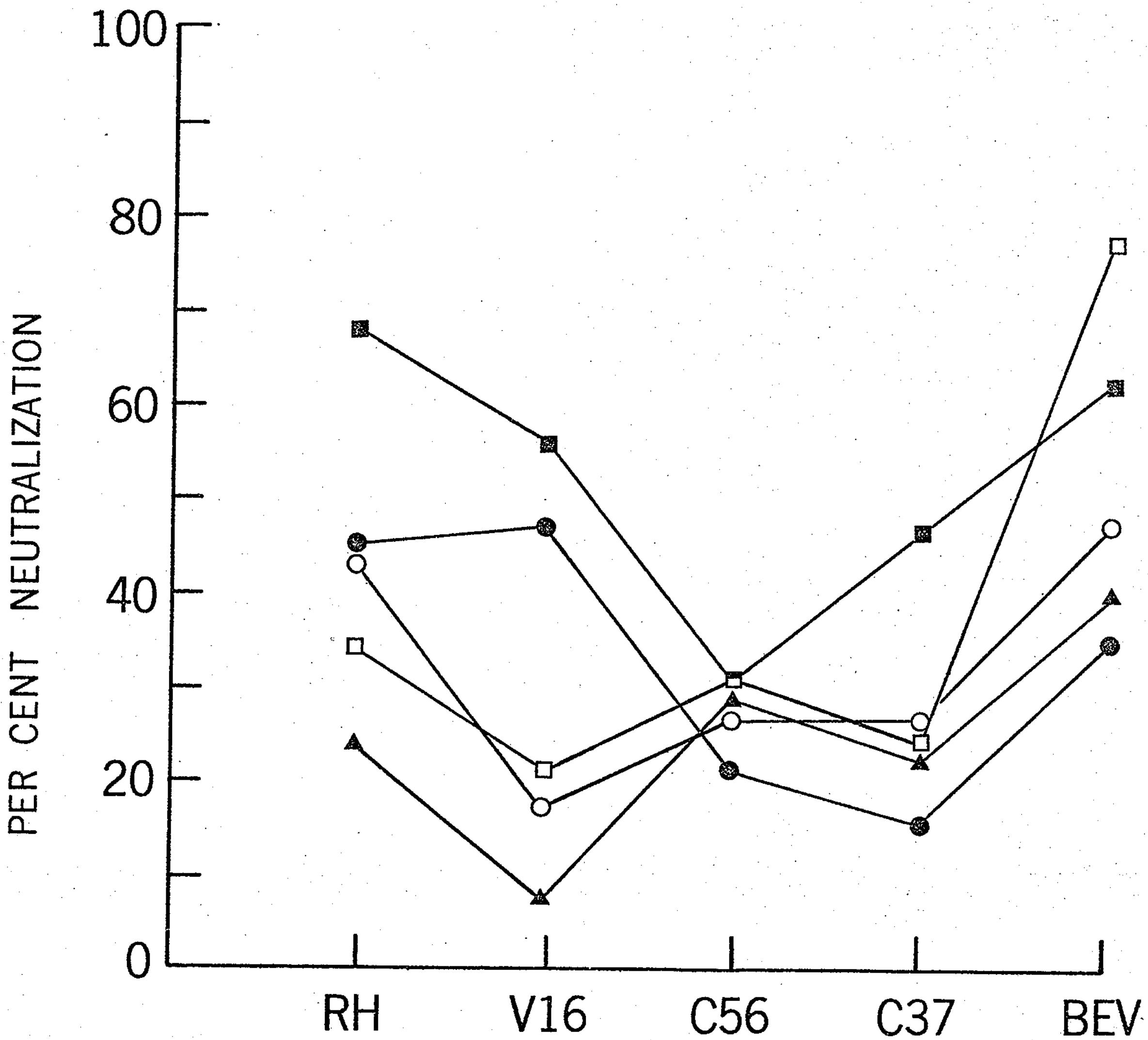


Figure 6B: Neutralization of five strains of Toxoplasma by each of the five strain-specific antisera in the dilution method - results of two experiments.

Graph showing neutralization antigen-wise in Experiment 1.

FIGURE 6C

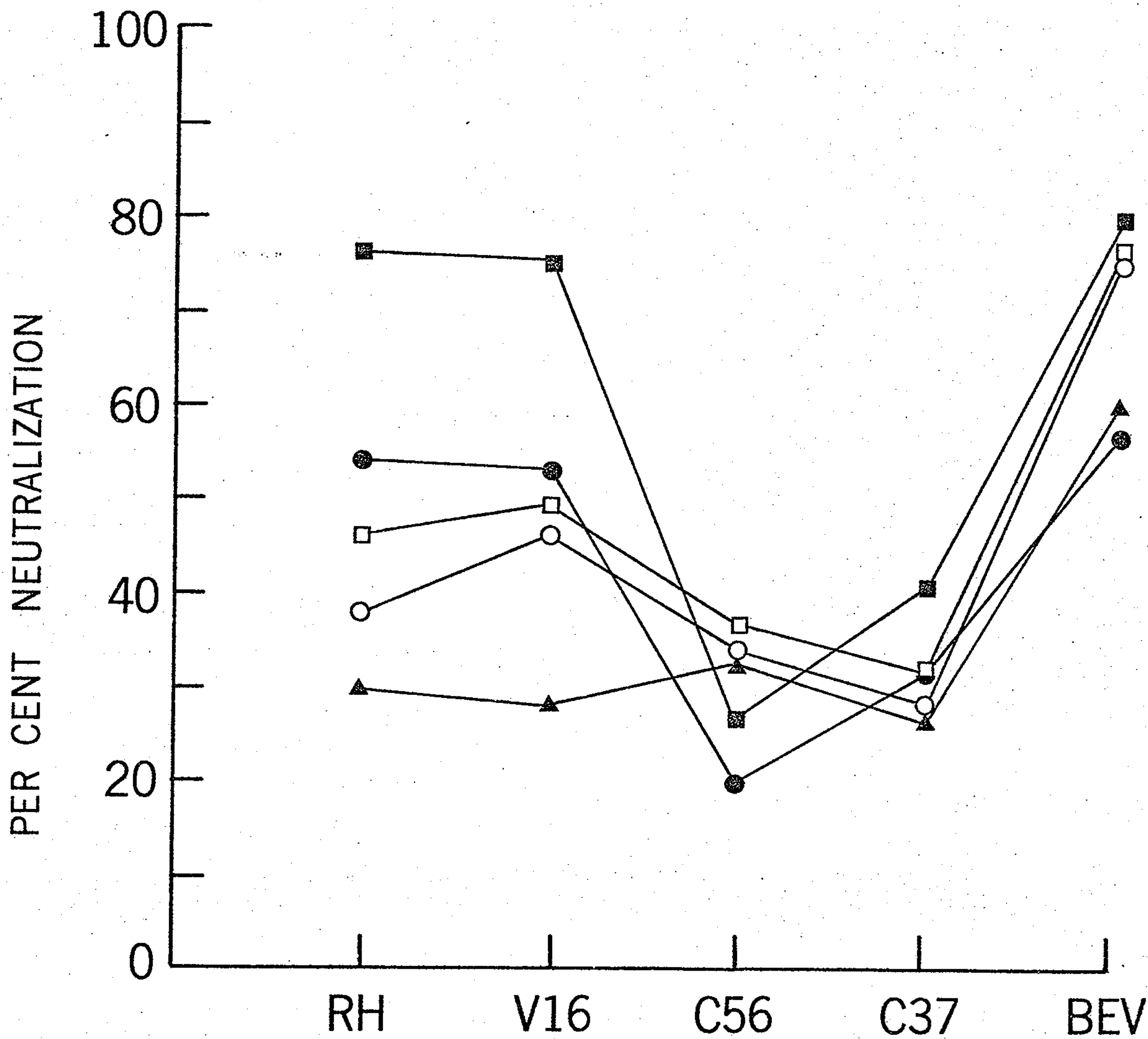


Figure 6C: Neutralization of five strains of Toxoplasma by each of the five strain-specific antisera in the dilution method - results of two experiments.

Graph showing neutralization antigen-wise in Experiment 2.

fraught with inaccuracy. Therefore, while an approximate count was made, it was not possible to vouch for the accuracy of the count and it follows therefore that the concentration of toxoplasma suspensions was not exactly the same in each and every experiment even as it was taken and contended to be at a particular concentration such as 4×10^6/ml. Thus it became necessary to investigate further into this inordinate variation in the results of repeated experiments by the dilution method over and above reasonable margins of errors that could be accounted for by biological variability of either toxoplasma or the mice in which they were passaged and by the experimental measurement error. Hence this experiment.

Four serial two-fold dilutions of toxoplasma (RH) were made in HBSS pH 7.7 such that each dilution contained approximately 8×10^6, 4×10^6, 2×10^6 and 1×10^6 toxoplasma/ml. Each dilution had a control and test system set up. Control system comprised 0.5 ml each of toxoplasma suspension in HBSS pH 7.7 of appropriate concentrations, HBSS pH 7.7, inactivated complement (guinea pig serum diluted 1-16) and preimmune rabbit serum. Test system was similar to the corresponding control except that 0.5 ml antiRH serum was taken in place of preimmune serum. Assay for plaques was made at zero and 8 hours after a dilution of 400-, 200-, 100-, and 50-fold for concentrations corresponding to respectively 8×10^6, 4×10^6, 2×10^6 and 1×10^6 per ml. The results are presented in figure 7A.

In a second similar experiment concentrations of toxoplasma ranging from about 10×10^6, 5×10^6, 2.5×10^6 per ml were taken and a dilution of 400-, 200-, and 100-fold respectively was carried out. In addition,

FIGURE 7A

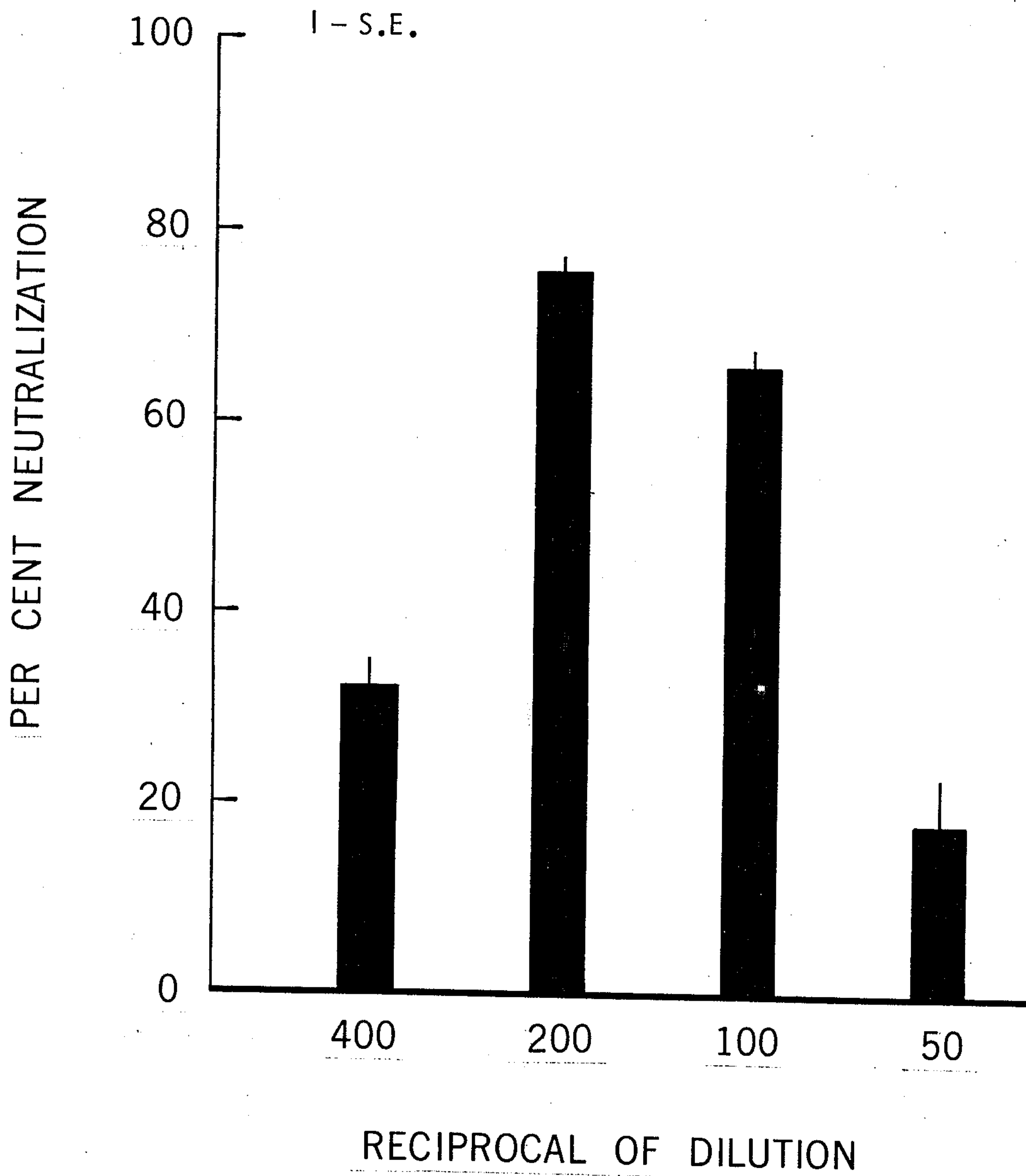


Figure 7A: Neutralization with varying concentrations of Toxoplasma(RH), and constant antiserum-and-inactivated complement by dilution method.

direct method was simultaneously set up at a concentration of about 2.5×10^4/ml of toxoplasma. The results of neutralization obtained in the experiments can be seen in figure 7B which reveals that neutralization varied in its proportion as the concentration of toxoplasma varied. In the dilution method neutralization was low at both high and low concentrations of toxoplasma and maximum neutralization was produced at an intermediate concentration or the optimum concentration of toxoplasma. In the direct method not involving dilution, the concentration of toxoplasma, per se, though 50 to 400 times lower than in any of the tubes in the dilution method, the neutralization obtained was slightly higher than the highest obtained in the dilution method. While the variation in neutralization was obviously due to reactivation or dissociation of toxoplasma on dilution, the proportion or degree of reactivation depended on the concentration of toxoplasma in relation to other components of the reaction system. The percentage of neutralization obtained in the direct method probably represents the neutralization that can be expected at the optimum zone in the dilution method if it were possible to have the exact concentration of toxoplasma required in this zone.

When neutralization of toxoplasma at various concentrations were compared, in the first experiment highly significant differences were observed between 1-200 and 1-400 dilution ($0.01 < p < 0.02$), between 1-200 and 1-50 dilutions ($p < 0.001$). The difference in neutralization at 1-200 and 1-100 dilutions was significant at five per cent level.

In the second experiment, there was no significant difference observed between direct and 1-200 dilution ($0.1 < p < 0.2$), between 1-200 and 1-400 dilutions ($0.2 < p < 0.3$) as well as between 1-200 and 1-100

FIGURE 7B

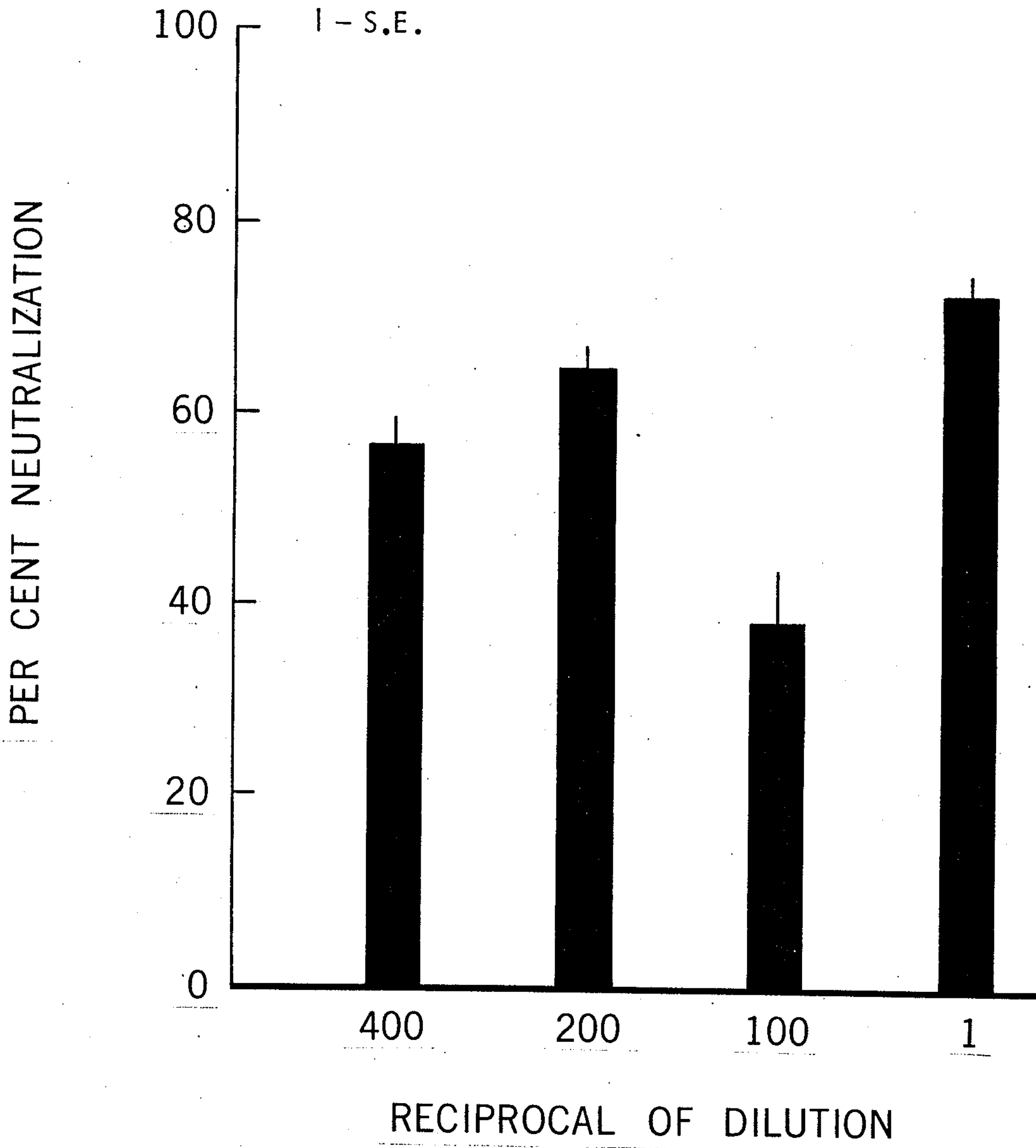


Figure 7B: Neutralization with varying concentrations of Toxoplasma(RH) and constant antiserum-and-inactivated complement by dilution method and comparison with direct method.

dilutions ($0.5 < p < 0.1$). However, the difference in neutralization obtained by direct method as compared to 1-100 dilution was highly significant ($p < 0.01$). Difference in neutralization by direct method and 1-400 dilution gave significant difference ($p < 0.05$).

Experiment 16

Neutralization with varying concentrations of purified toxoplasma, and constant antiserum-and-inactivated complement by dilution method.

In order to find out if any soluble factors in toxoplasma suspensions used in neutralization experiments act as haptens and thus interfere in neutralization or cause dissociation of toxoplasma on dilution, toxoplasma(RH) was purified by differential centrifugation as described in materials and methods. Plaque assay was carried out on Vero monolayers in T-flasks incubated in an ordinary incubator at 37°C instead of in petri dishes in CO_2 incubator. Each component of the reaction system was taken in 0.2 ml amounts instead of 0.5 ml. Concentrations of purified toxoplasma in the dilutions employed varied from about 2×10^7/ml, 1×10^7/ml, 5×10^6/ml, 2.5×10^6/ml, and 1.25×10^6/ml and the corresponding dilution factors were 400-, 200-, 100-, 50-, and 25-fold respectively. Concentrations of toxoplasma for the direct method was about 5×10^4/ml.

From the results shown in figure 8 it is apparent that purification of toxoplasma did not alter the neutralization picture. In other words, there does not seem to be any soluble factor in the toxoplasma suspension influencing dissociation on dilution since the variation in neutralization followed a similar pattern with purified toxoplasma at varying concentrations as with toxoplasma which was not purified.

Highly significant differences ($p < 0.001$) were observed when the proportion of neutralization in the direct method was compared with those obtained in 1-400, 1-100, 1-50, and 1-25 dilutions. There was no significant difference in neutralization in the direct method when compared to that obtained in 1-200 dilution.

Experiment 17

Comparison of neutralization of purified toxoplasma by antiserum alone by dilution and direct methods

In previous studies (Shettigara, 1971) it was not possible to obtain any significant neutralization of toxoplasma(RH) by rabbit antiRH serum alone when assayed by dilution method but significant neutralization was observed on direct assay. In order to ascertain if in the absence of guinea pig complement, purification of toxoplasma would influence neutralization by its antiserum on dilution as well as on direct assay, neutralization of purified toxoplasma(RH) was carried out in the presence of rabbit antiRH serum alone both by direct and by dilution methods and assayed for plaques in T-flasks at zero and 8 hours.

In the dilution method, control A tube contained 0.2 ml of toxoplasma (RH) suspension in HBSS pH 7.7 at a concentration of about 10×10^6/ml and 0.4 ml HBSS buffer pH 7.7. In control tube B, 0.2 ml rabbit preimmune serum was taken. Test A tube was similar to control A tube. Test B tube contained 0.2 ml rabbit antiRH serum.

In the direct method control and test system tubes were identical to those in the dilution method, except that the concentration of toxoplasma suspension was about 5×10^5/ml. 0.2 ml of the neutralization mixture

FIGURE 8.

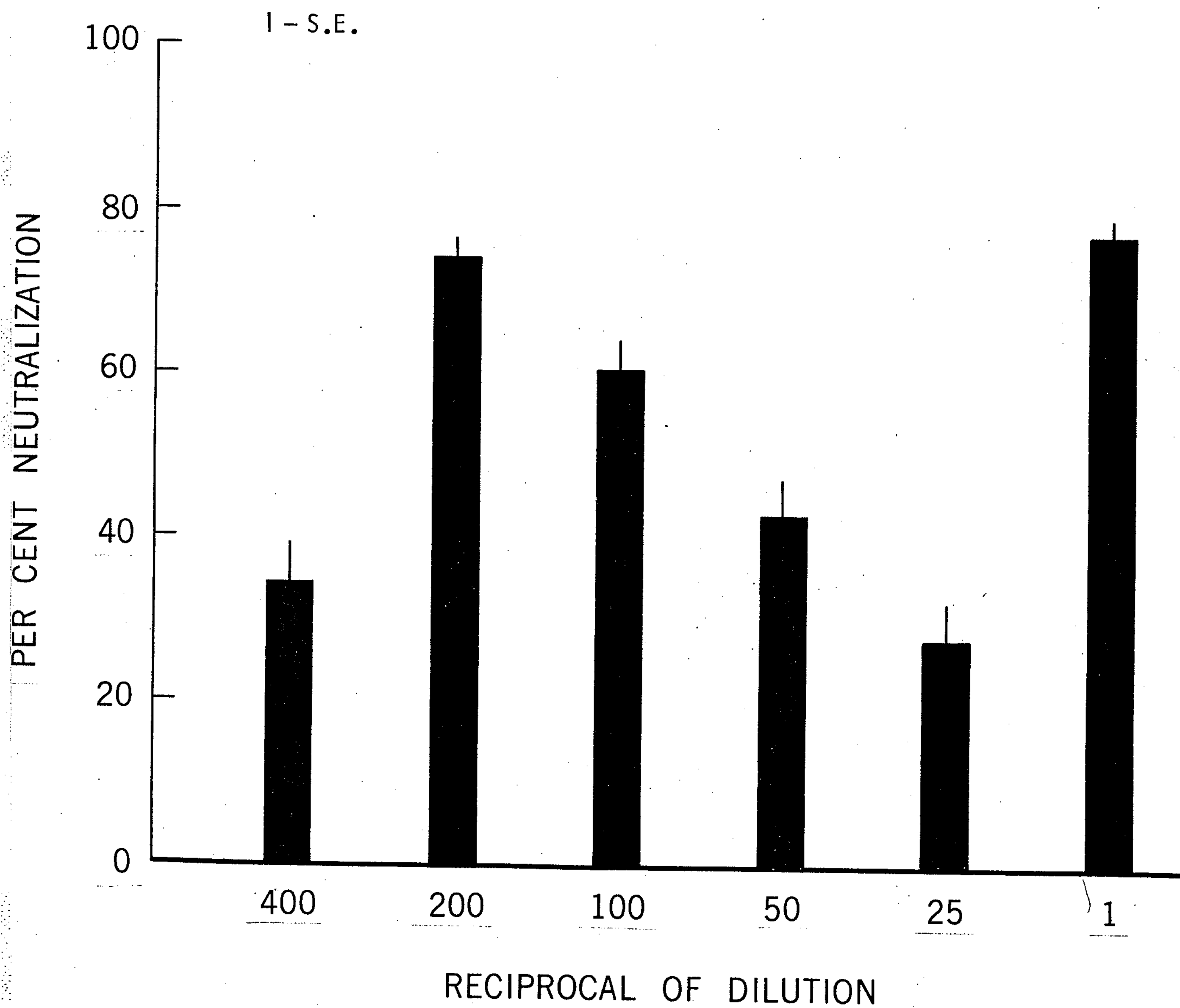


Figure 8. Neutralization with varying concentrations of purified Toxoplasma(RH) and constant antiserum-and-inactivated complement by dilution method and comparison with direct method.

was assayed directly on single monolayers in T-flasks at zero hour and on replicate monolayers at 8 hours in the direct method, and after 1-200 dilution in the dilution method.

Once again the results given in table 19 indicated that purification of toxoplasma did not alter the neutralization or dissociation events.

Experiment 18

Neutralization of the five strains of purified toxoplasma by their homologous and heterologous antisera by direct method.

In view of the observed dissociation of toxoplasma on dilution and of the difficulty to ascertain with accuracy the count of toxoplasma to obtain a dependable optimum concentration every time if dilution method were to be employed, it was decided to apply the direct method wherein, in the absence of dilution there was no dissociation of toxoplasma.

Purified toxoplasma suspension in HBSS pH 7.7 at a concentration of approximately 5 x 10^5/ml, HBSS pH 7.7, and inactivated complement (guinea pig serum diluted 1-16) were each taken in 0.2 ml amounts and rabbit antiserum against individual strains of toxoplasma for the test system as well as rabbit preimmune serum for the control system were also taken in 0.2 ml volumes. 0.2 ml of the neutralization mixture was assayed directly on single Vero monolayers at zero hour, and, on replicate monolayers in petri dishes at eight hours.

The result of neutralization obtained in respect of each of the five strains of toxoplasma by its homologous and heterologous antisera are given in figures 9-A, -B & -C. Neutralization experiment for each strain-serum combination was repeated on similar lines as above on a different occasion to obtain reproducible results, there being no significant difference between the corresponding neutralization values in the two experi-

TABLE 19

COMPARISON OF NEUTRALIZATION OF PURIFIED TOXOPLASMA (RH) BY ANTIRH SERUM (WITHOUT COMPLEMENT) BY DILUTION AND DIRECT METHODS.

TIME	1 - 200 DILUTION			DIRECT		
	C *	T **	%N +	C *	T **	%N +
0 Hours	256 x	258	-	259	257	-
	160,166	165,158		163,160	98,93	
8 Hours	163 ¢	161	-	161	93	42.24%

* Control system in the dilution method comprised 0.2ml suspension of toxoplasma (RH) in HBSS pH 7.7 at a concentration of about 10×10^6/ml, 0.4ml HBSS buffer, and 0.2ml rabbit preimmune serum. Control system in the direct method was similar except the concentration of toxoplasma was about 5×10^5/ml.

**Test systemsin the dilution and the direct methods were identical to the corresponding control system except that in place of rabbit preimmune serum, rabbit antiRH serum was employed.

+ Percent neutralization.

x Number of plaques in single plates.

¢ Average number of plaques in two replicates.

FIGURE 9A

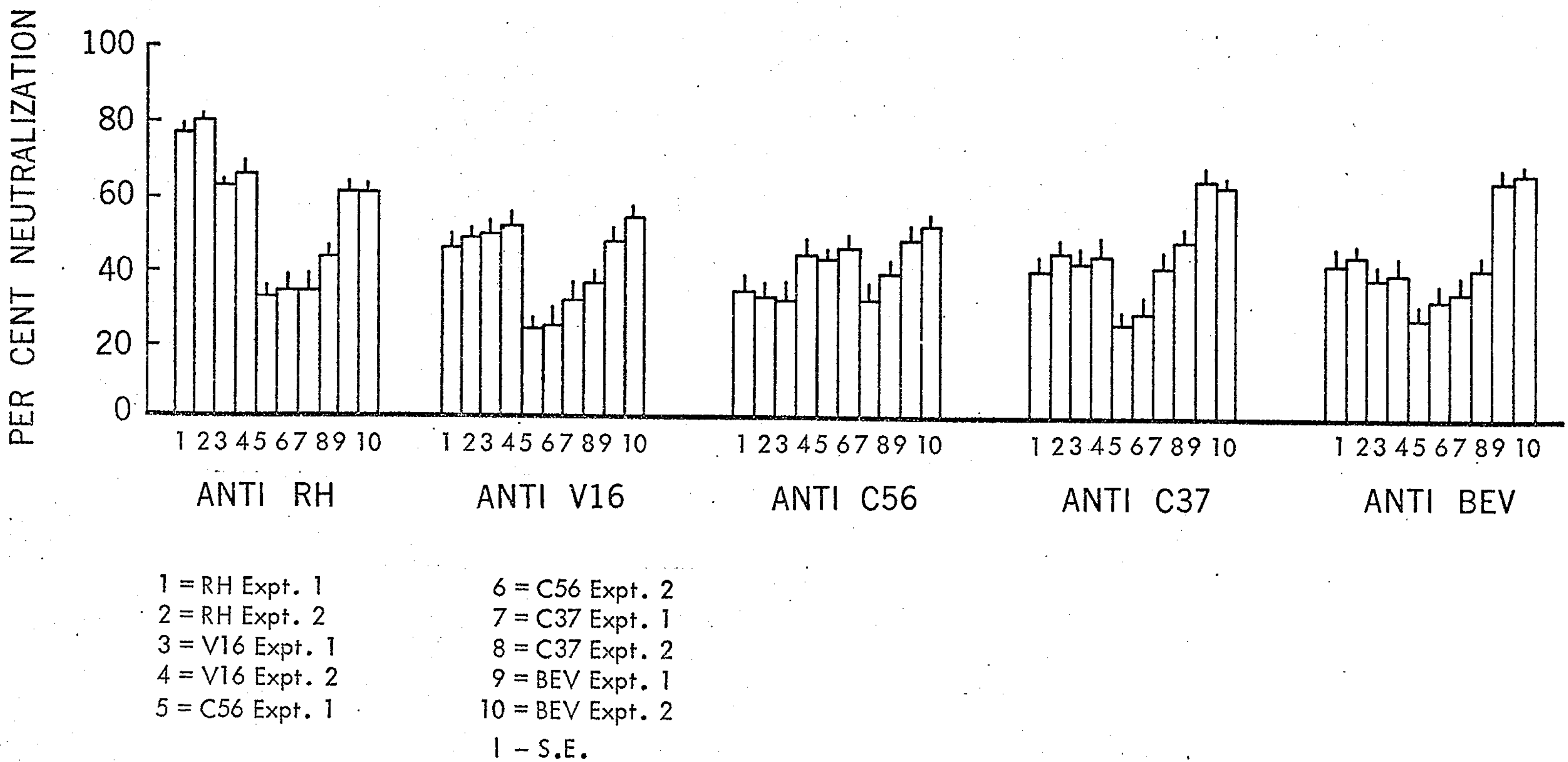


Figure 9. Neutralization of each strain of purified Toxoplasma by its homologous and heterologous antisera by direct method.

Fig. 9A: Histogram showing neutralization antiserum-wise in Experiment 1 and 2.

FIGURE 9B

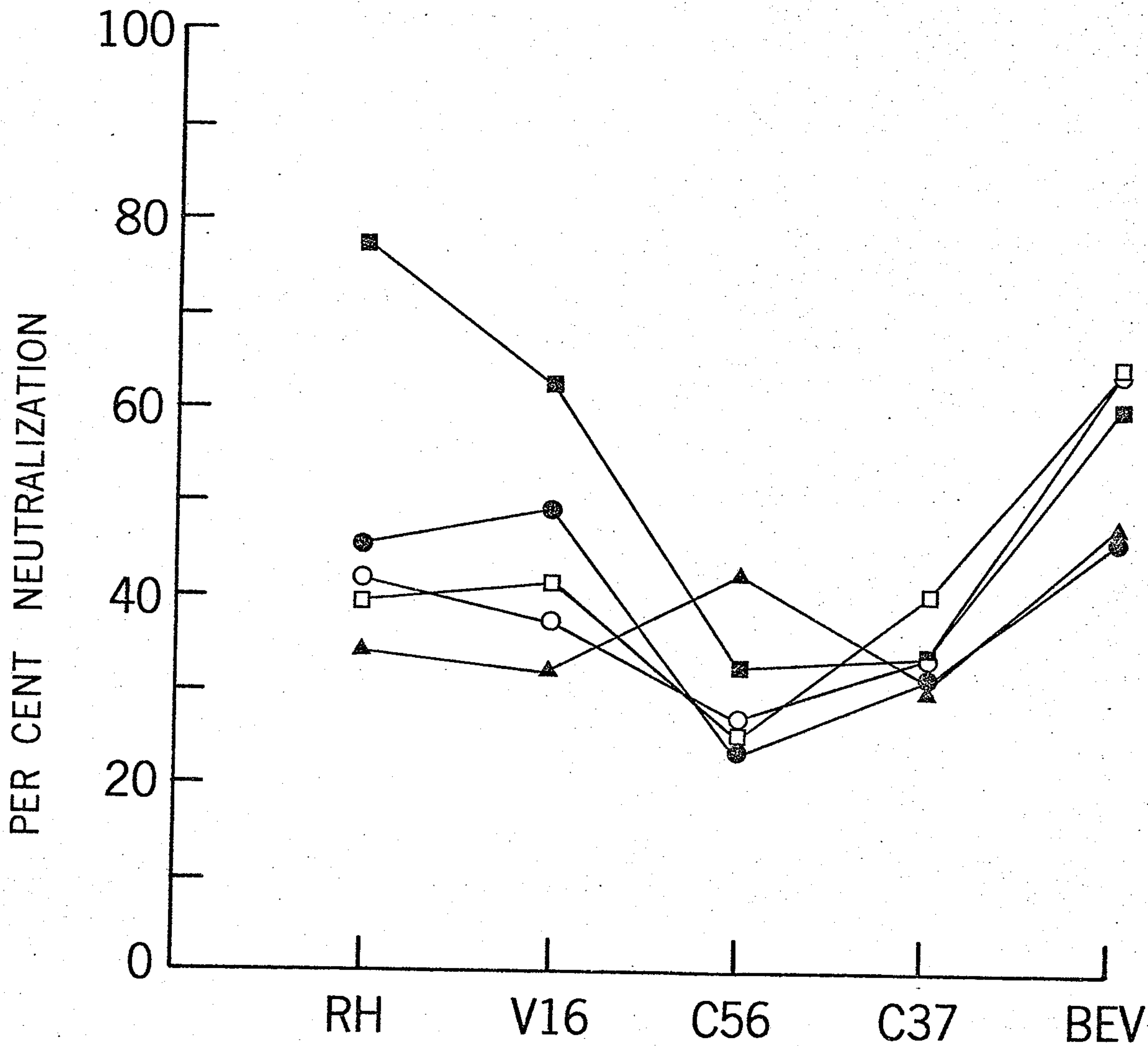


Figure 9B: Neutralization of each strain of purified Toxoplasma by its homologous and heterologous antisera by direct method.

Graph showing neutralization antigen-wise in experiment 1.

FIGURE 9C

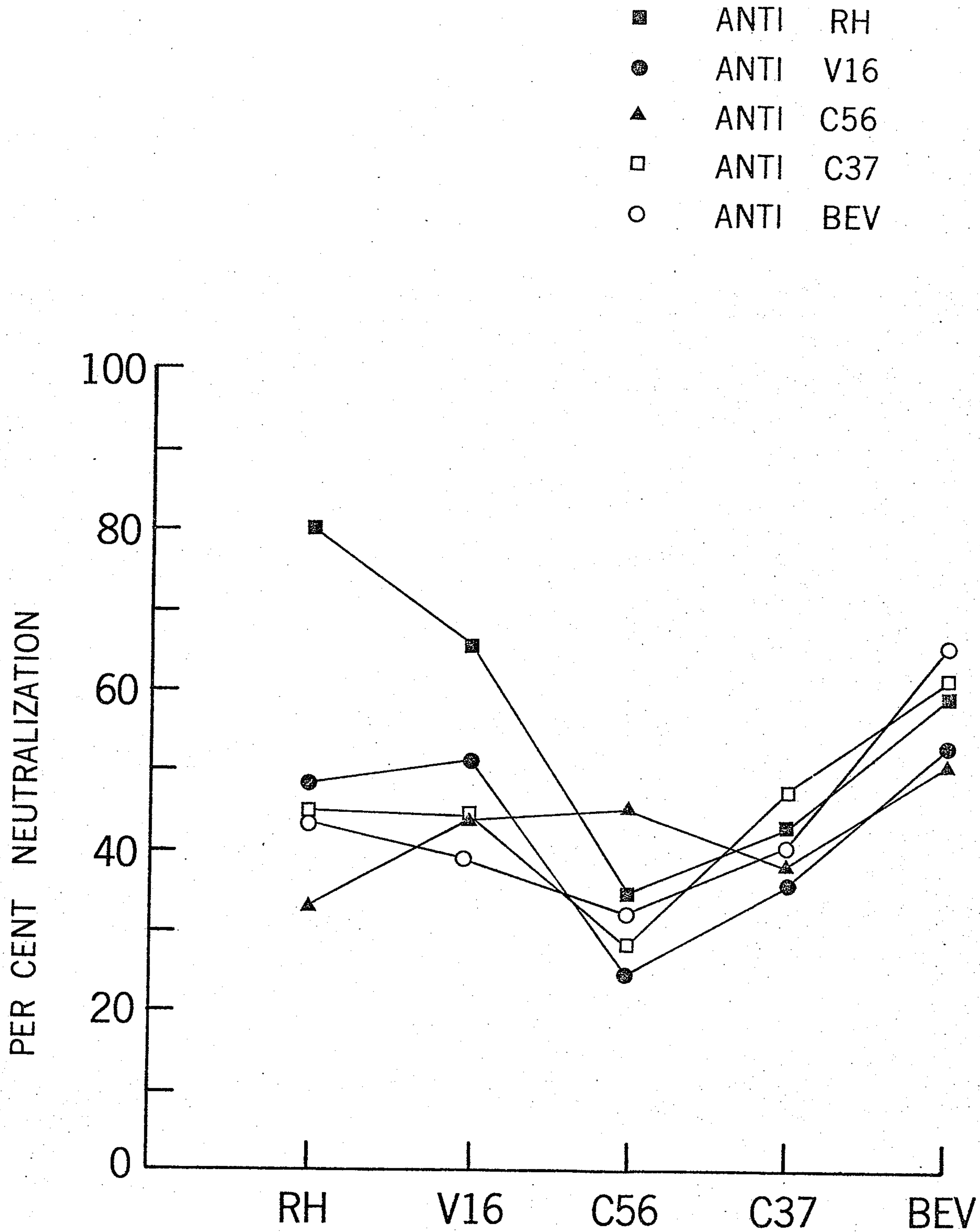


Figure 9C: Neutralization of each strain of purified Toxoplasma by its homologous and heterologous antisera by direct method.

Graph showing neutralization antigen-wise in experiment 2.

ments.

Combining the results of the two experiments and analysing it antigen-wise, employing all combination of two strains by two-way contingency revealed that there was no significant difference between C56 and C37 ($0.05 < p < 0.10$), and between C37 and Beverley ($0.20 < p < 0.30$). There was significant difference between RH and V16 ($0.025 < p < 0.05$). Highly significant differences were observed between the rest, namely, RH and C56, RH and C37, RH and Beverley, V16 and C56, V16 and C37, V16 and Beverley, and C56 and Beverley ($p < 0.005$ in each).

When the antisera were analyzed for significance of difference from each other, borderline significance was observed between antiV16 and anti-C37 ($0.05 < p < 0.10$) and no significant difference between antiC37 and antiBeverley ($p > 0.50$). Significant differences were seen between antiV16 and antiBeverley ($0.01 < p < 0.025$) and highly significant difference ($p < 0.005$) among the rest, namely, antiRH and antiV16, antiRH and antiC56, antiRH and antiC37, antiRH and antiBeverley, antiV16 and antiC56, antiC56 and antiC37 and antiC56 and antiBeverley. Considering the analyses in respect of both the antigens and the antisera together, it was only between C37 and Beverley and their corresponding antisera, namely antiC37 and antiBeverley was there no significant difference at the 10% level of significance. At an arbitrary level of 10% significance it can, therefore, be concluded that it would not be possible to differentiate either C37 from Beverley or antiC37 serum from antiBeverley serum. In other words, C37 and Beverley strains can be considered as belonging to one group and the others, namely, RH, V16, and C56 as belonging to three separate groups, each being distinctly different from the other in reciprocal neutralization.

Ashe and Scherp (1963) employed 90% similarity as the criterion for a preliminary grouping of Herpes simplex strains using normalized kinetic (NK) values. When this conventional method of comparing the normalized values in respect of each strain against the five different antisera was attempted making use of the normalized neutralization values as shown in table 20, it became clear that RH, V16, C56, C37 and Beverley were each distinctly different from each other at the 90% level of similarity, However, the closest reciprocal similarity was seen between C37 and Beverley, each respectively giving normalized neutralization values of 100 and 85 and 97 and 100. In the conventional method the strength of relatedness is confined to NK values ranging from 100 to 90 at the 90% level. In the Chi-square test employed in the present study, the relatedness of the whole spectrum of neutralization of each antigen by all the five individual antisera is examined and hence the difference between the two tests.

V16 strain was seen to give a higher NK value with antiRH (heterologous) serum than with antiV16 (homologous) serum, an unusual deviation from the normal pattern of neutralization.

TABLE 20

RECIPROCAL PERCENTAGE NEUTRALIZATION AND NORMALIZED NEUTRALIZATION RESULTS OF FIVE STRAIN SPECIFIC TOXOPLASMA ANTISERA WITH THE RESPECTIVE STRAINS OF TOXOPLASMA

Strain of Toxoplasma	Antiserum	Per Cent Neutralization	Normalized Neutralization
RH	anti RH	79	100
	anti V16	47	59
	anti C56	33	42
	anti C37	42	53
	anti Beverley	43	54
V16	anti RH	64	128
	anti V16	50	100
	anti C56	38	76
	anti C37	43	85
	anti Beverley	38	76
C56	anti RH	34	77
	anti V16	24	55
	anti C56	44	100
	anti C37	27	61
	anti Beverley	30	68
C37	anti RH	39	88
	anti V16	34	77
	anti C56	35	79
	anti C37	44	100
	anti Beverley	37	85
Beverley	anti RH	60	93
	anti V16	50	77
	anti C56	50	76
	anti C37	63	97
	anti Beverley	65	100

VIRULENCE TESTS:

To determine the virulence of each of the five strains of toxoplasma, namely, RH, V16, C56, C37, and Beverley, the average lethal dose or the fifty per cent lethal dose (LD 50) of each strain of toxoplasma for mice in terms of plaques in Vero monolayers and the average response time or the fifty per cent response time-to-death (RT50) in mice in respect of each strain were determined following the procedures outlined in materials and methods. At least two such experiments were performed for each strain of toxoplasma on two different occasions. (For details of plaque numbers, percentage of death and time-to-death of mice, see Appendix N to W).

A. RH Strain

In the first experiment, 12 serial two-fold dilutions of the toxoplasma(RH) suspension were made in PBS-A with 2% calf serum, plus PSF. Assay for plaques for each dilution was carried out by inoculating 0.5 ml of each dilution into each of five Vero monolayers in petri dishes. Of the 12 dilutions, the last seven were used to inoculate Swiss White mice, groups of ten mice per dilution, 0.5 ml of the dilution being injected intraperitoneally into each mouse.

Controls comprised two groups of 10 mice each. One group of mice was injected with the suspending fluid-PBS-A with 2% calf serum, plus PSF, 0.5 ml per mouse, given intraperitoneally. The second group of ten mice were kept under observation uninoculated. Both the test and the control groups of mice were observed for twenty days.

In the second experiment, 10 serial two-fold dilutions were made for inoculation into monolayers and the last 9 dilutions were used for inject-

ing the mice as above.

Values of LD50 and RT50 in each of the experiments were obtained on analysis of the results by Probit and Rankit transformations respectively, as detailed under materials and methods and shown in figures 10 and 11. The values of LD50 and RT50 observed in the first experiment were 0.369 and 9.14 and in the second experiment 0.671 and 9.10 respectively.

Times-to-death in test mice ranged from 8 to 11 days in the first experiment and 8 to 10 days in the second.

B. V16 strain:

In the first experiment, 12 serial two-fold dilutions of V16 suspension were applied for plaque assay in Vero monolayers and the last eight dilutions were injected into mice.

In the next experiment, the dilutions were 11 for inoculation onto monolayers and the last 9 for injecting mice.

Deaths in test mice were encountered from 8 to 14 days in the first experiment and 8 to 13 days in the second.

LD50 for V16 strain in the two experiments were 0.697 and 0.526 and RT50 values were 10.54 and 10.52 (see figures 12 and 13).

C. C56 strain:

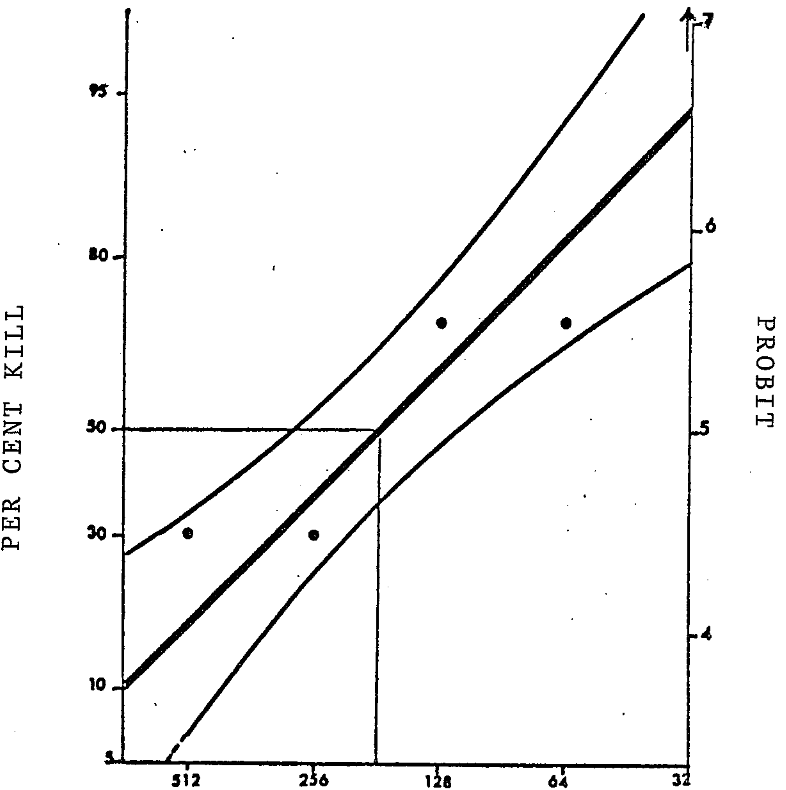
In both the experiments 13 serial two-fold dilutions of C56 strain were used for plaque assay and the last 10 dilutions were used for injecting mice.

Times-to-death in injected mice extended from day 9 to day 13 in both the experiments.

FIGURE 10

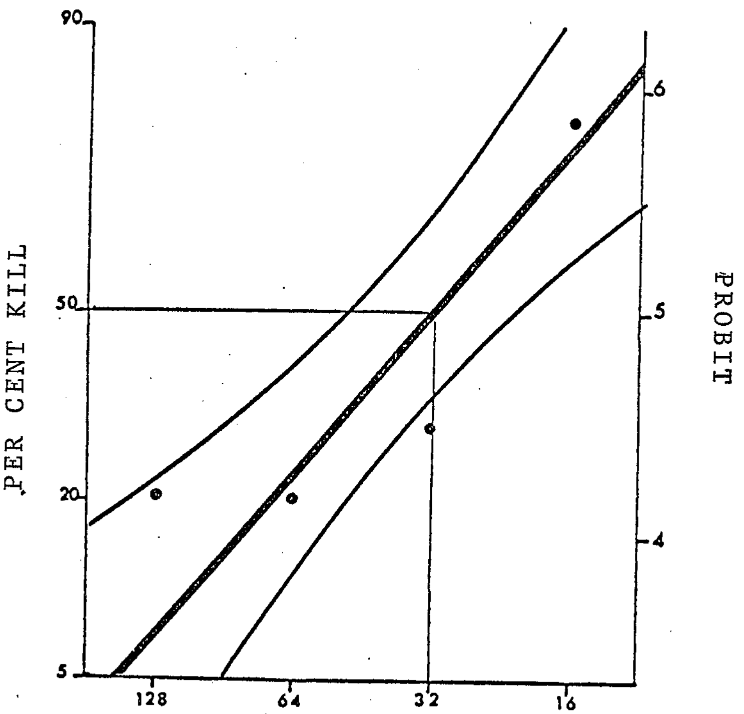
Estimation of ED50 dilution of RH-strain as a preliminary step for deriving the LD50 in plaque counts.

RH - Experiment 1



ED50 dilution = 0.0055
Estimated count of plaques in 0.5 ml
of undiluted RH suspension = K = 66.880
LD50 = 0.0055 x 66.880 = 0.369

RH = Experiment 2



ED50 dilution = 0.030
Estimated count of plaques in 0.5 ml
of undiluted RH suspension = K = 21.972
LD50 = 0.0306 x21.972 = 0.671

RH EXPERIMENT 1

RANKITS

LOG DAYS

DIL1 ×
DIL2 •
DIL3 o
DIL5 ▾

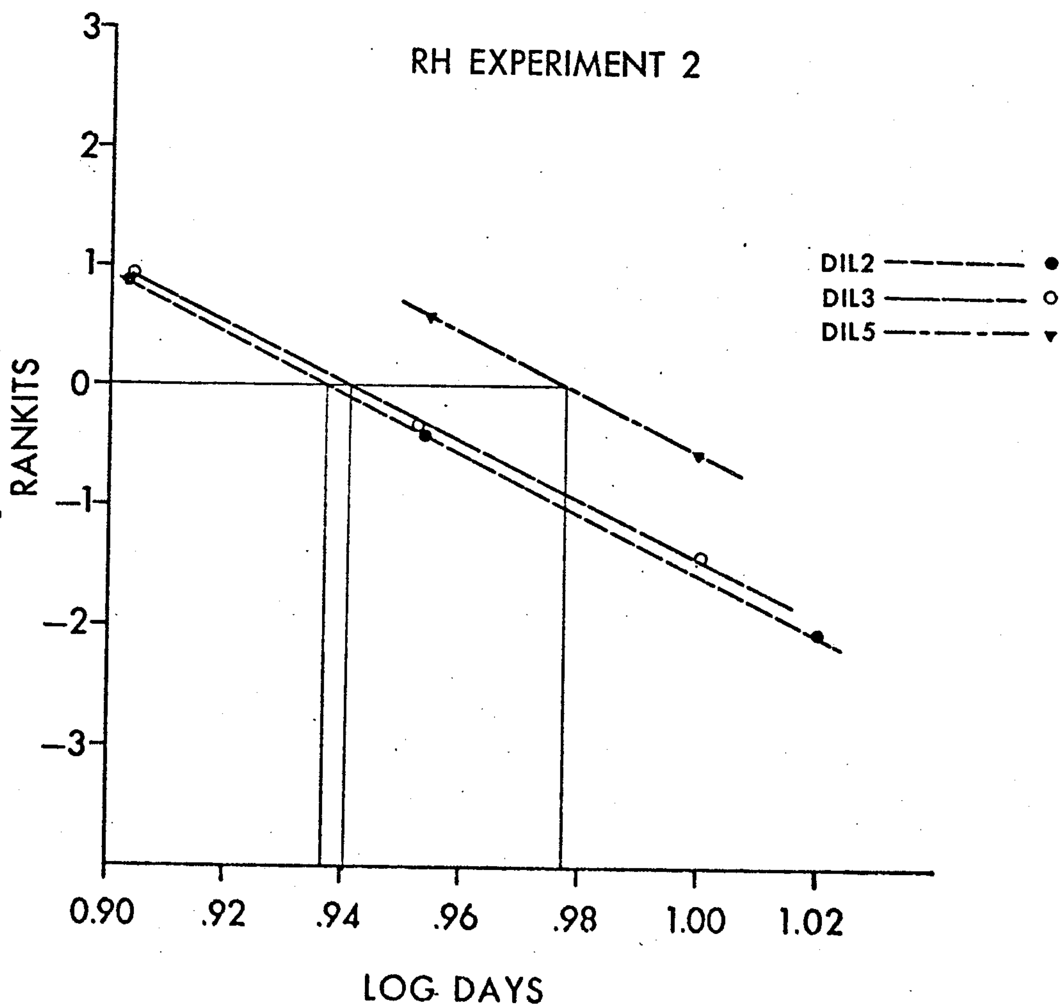


Figure 11: Estimation of RT50 of RH strain in terms of days by Rankit Transformation,
Experiment 1.
Experiment 2.

In the two experiments LD50 values of 0.924 and 0.664 and RT50 values of 11.12 and 10.33 were obtained and can be seen in figures 14 and 15 respectively.

D. C37 strain:

In the first experiment, 13 two-fold dilutions of C37 suspensions were inoculated onto monolayers whereas in the following experiment, 11 such dilutions were used. For injecting mice the last 10 dilutions and the last 9 dilutions respectively were used.

Times-to-death in mice injected ranged from 10 to 15 days in both the experiments.

LD50 values obtained for C37 were 1.615 and 1.750 and RT50 values were 12.82 and 11.97 in the two experiments (vide figures 16 and 17 respectively).

E. Beverley strain:

11 and 10 serial two-fold dilutions of Beverley strain suspension were used for plaque assay in the initial and the second experiments respectively and the last 9 and 8 dilutions respectively for injecting mice.

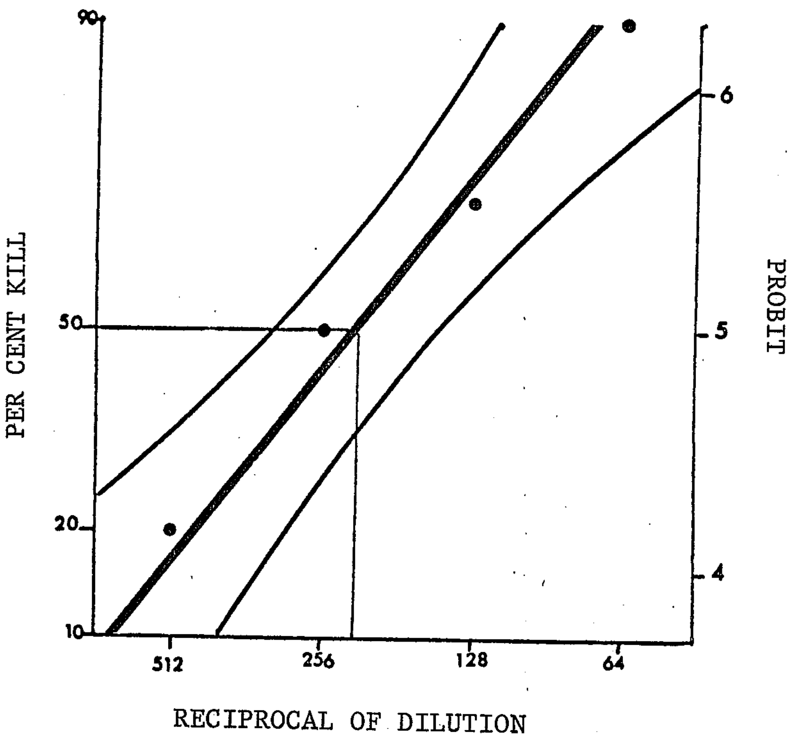
Times-to-death in test mice varied from 10 to 15 days in the first experiment and 10 to 16 days in the second experiment.

The two values of LD50 in respect of Beverley strain were 0.966 and 2.314 (figure 18) and the corresponding RT50 values were 11.66 and 12.97 (figure 19).

FIGURE 12

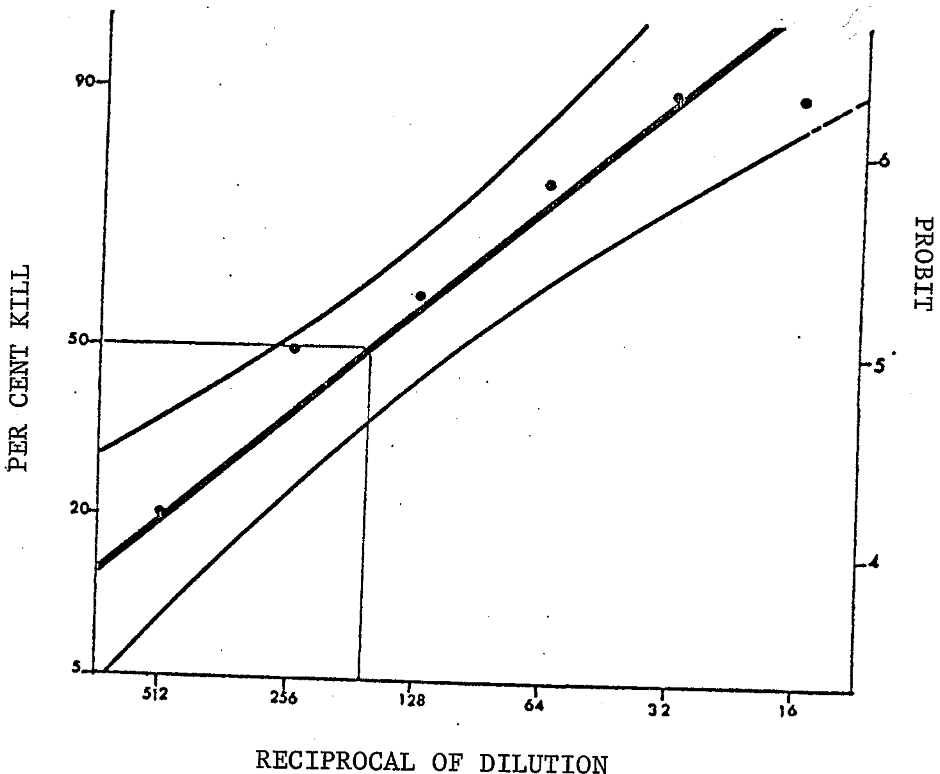
Estimation of ED50 dilution of V16 strain as a preliminary step for deriving the LD50 in plaque counts.

V16 - Experiment 1

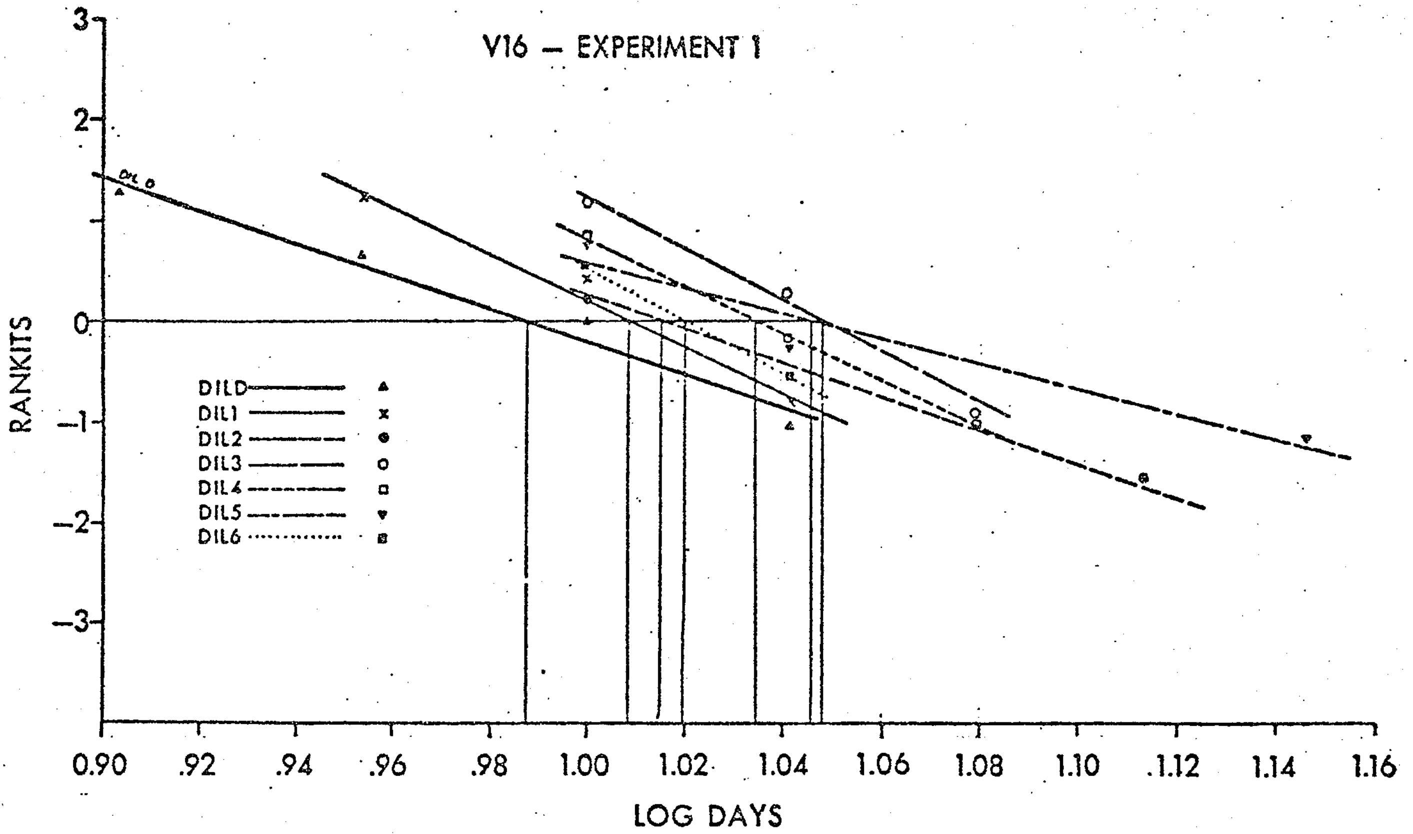


ED50 dilution = 0.0045
Estimated count of plaques in 0.5 ml
of undiluted V16 suspension = K = 154.413
LD50 = 0.0045 x 154.413 = 0.697

V16 - Experiment 2



ED50 dilution = 0.0059
Estimated count of plaques in 0.5 ml
of undiluted V16 suspension = K = 88.568
LD50 = 0.0059 x 88.568 = 0.526



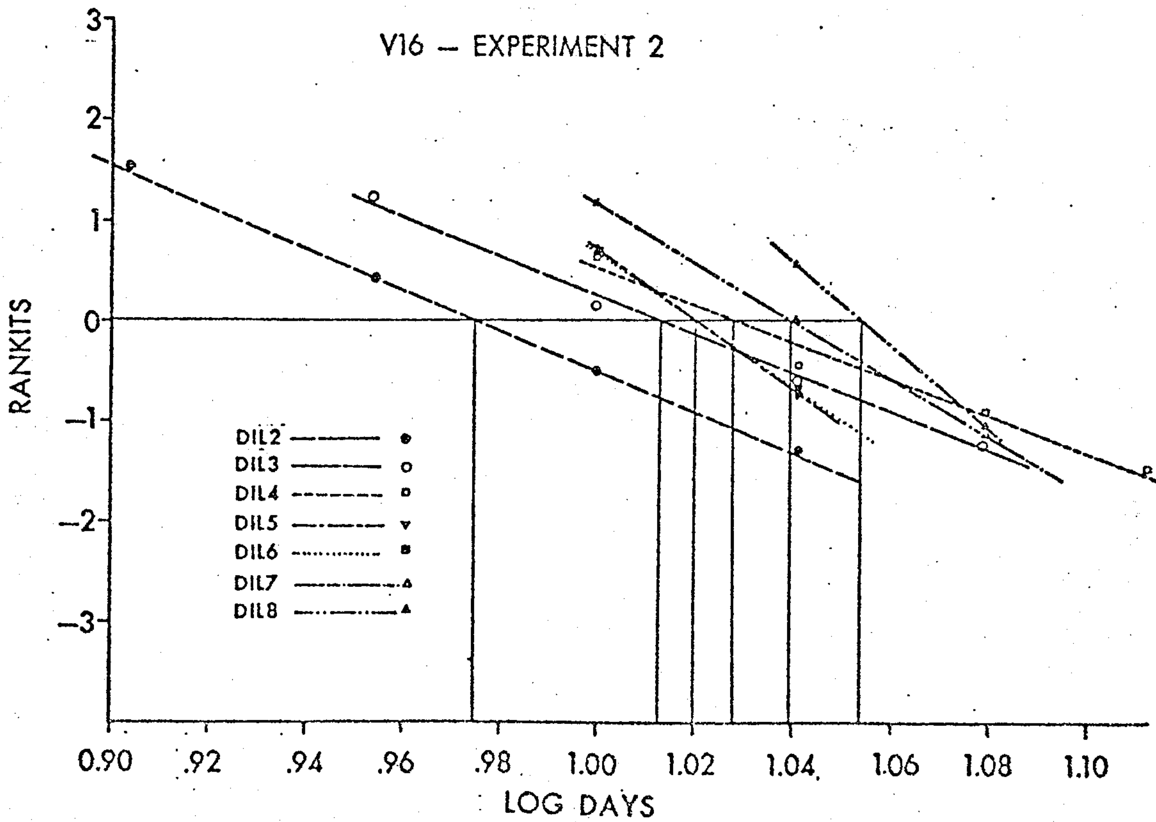
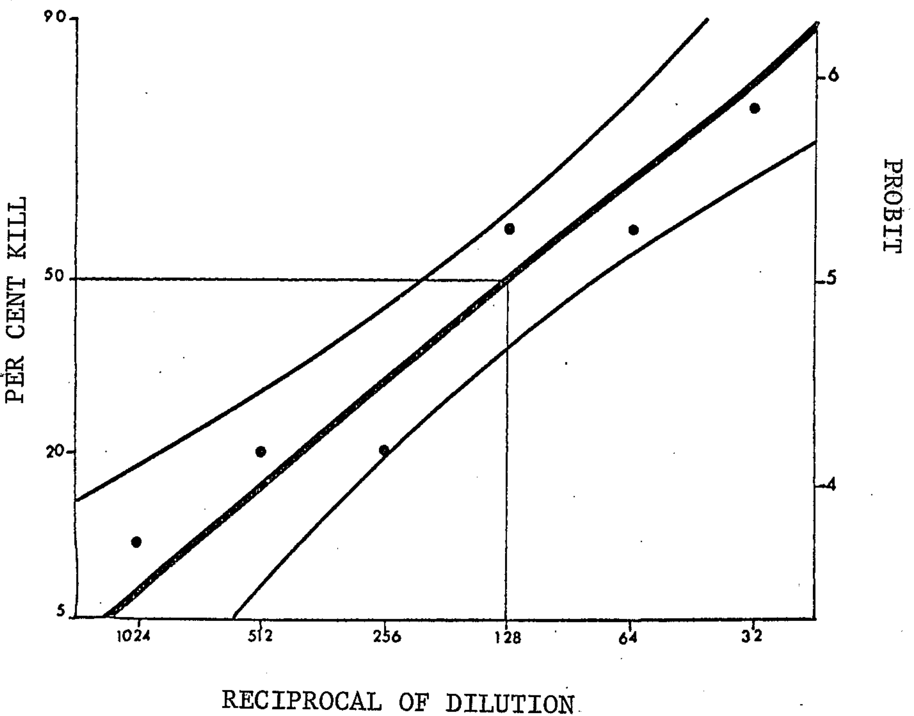


Figure 13: Estimation of RT50 of V16 strain in terms of days by Rankit Transformation.

FIGURE 14

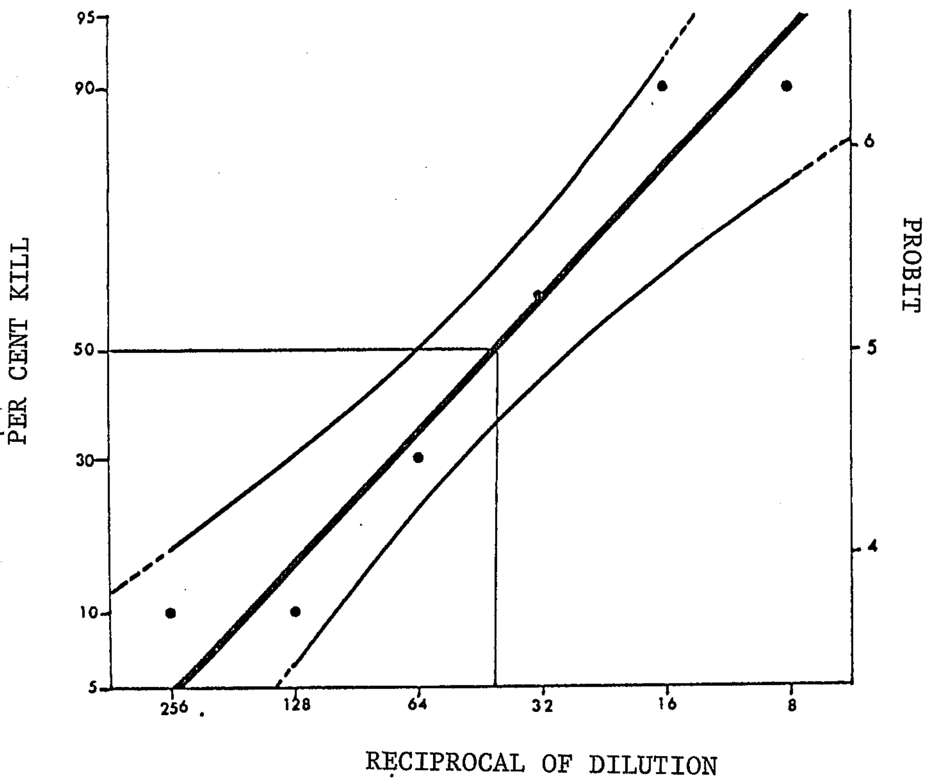
Estimation of ED50 dilution of C56 - strain as a preliminary step for deriving the LD50 in plaque counts.

C56 - Experiment 1

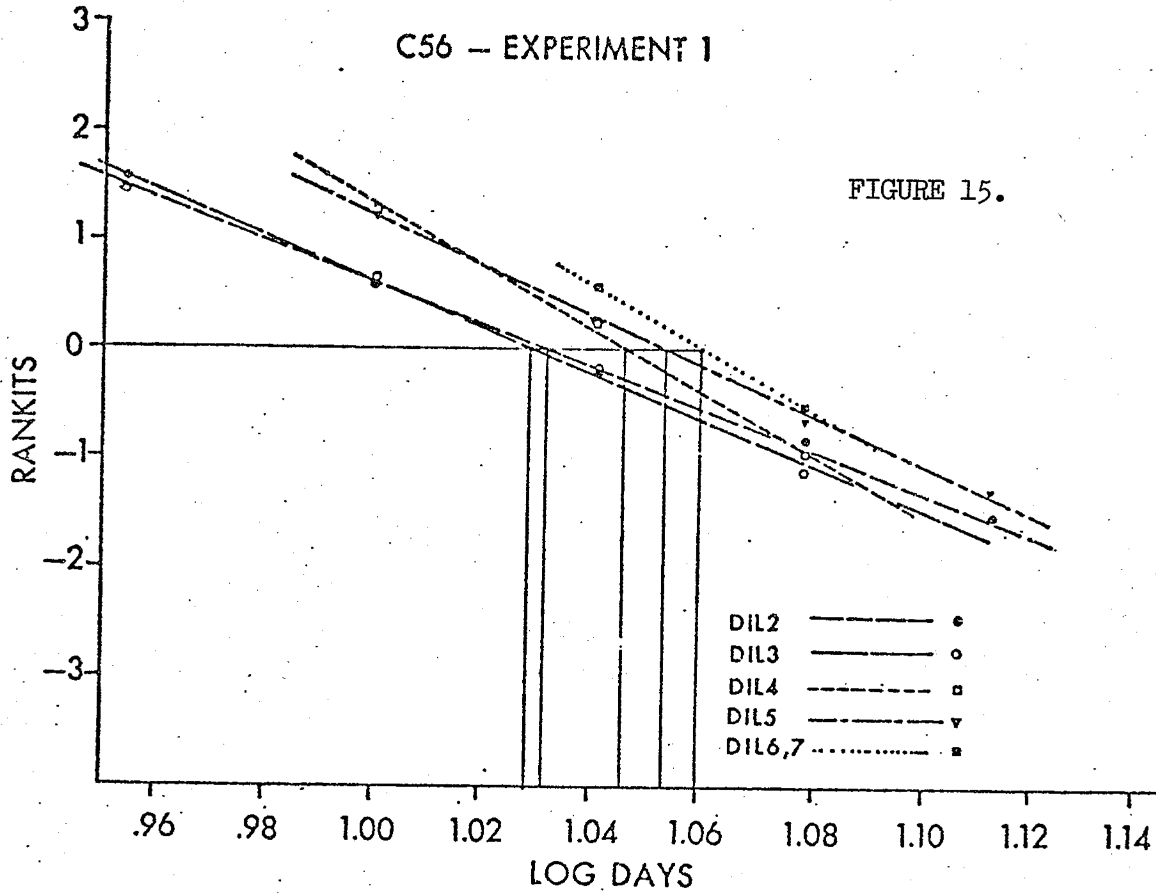


ED50 dilution = 0.0078
Estimated count of plaques in 0.5 ml
of undiluted C56 suspension = K = 117.748
LD50 = 0.0078 x 117.748 = 0.924

C56 - Experiment 2



ED50 dilution = 0.0239
Estimated count of plaques in 0.5 ml
of undiluted C56 suspension = K = 27.789
LD50 = 0.0239 x 27.789 = 0.664



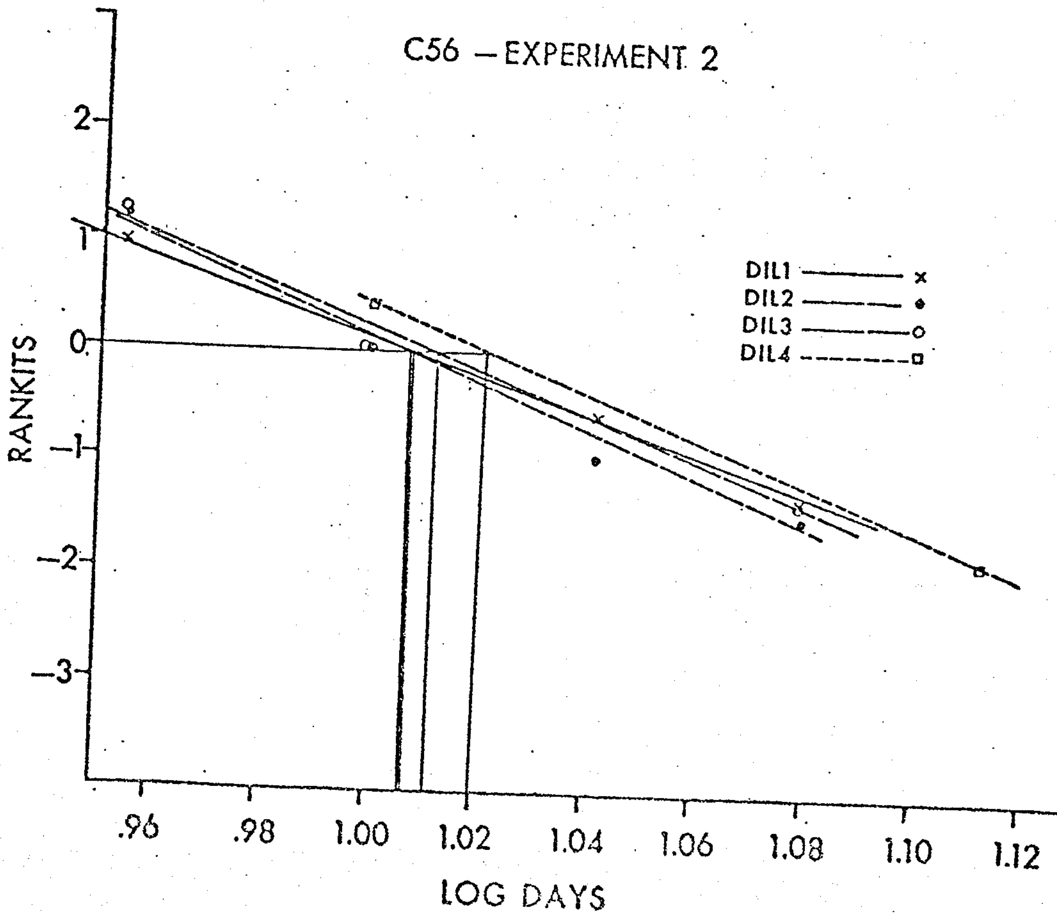
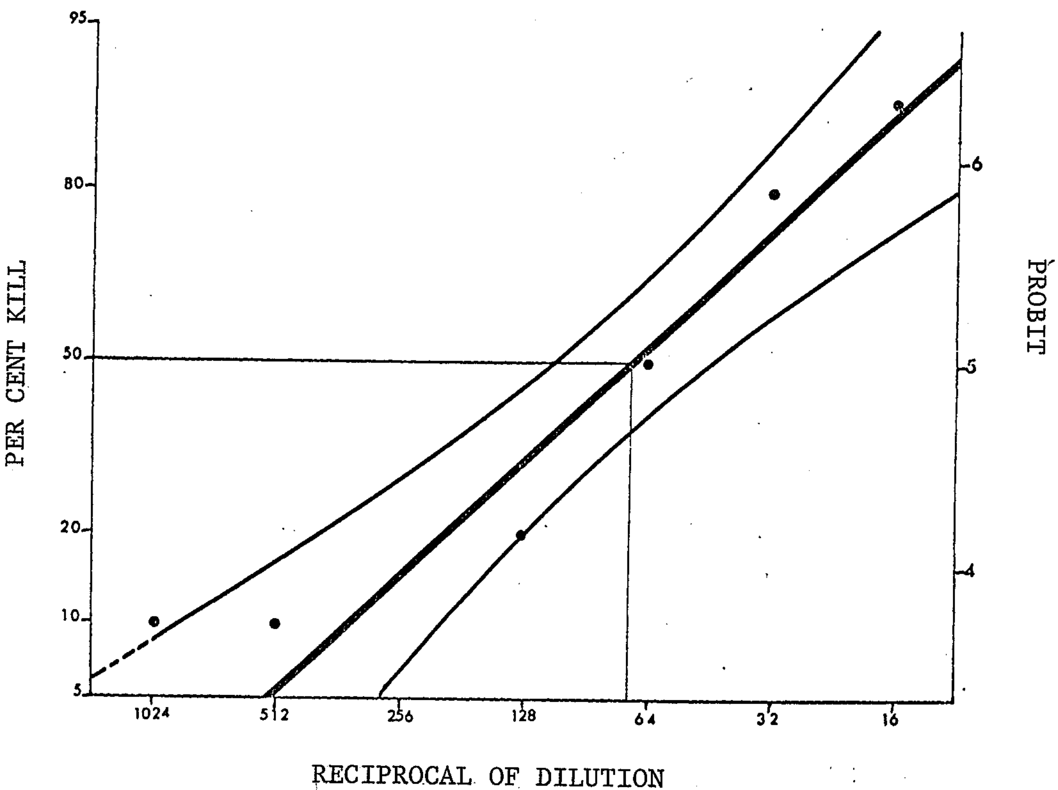


Figure 15: Estimation of RT50 of C56 strain in terms of days by Rankit Transformation.

FIGURE 16

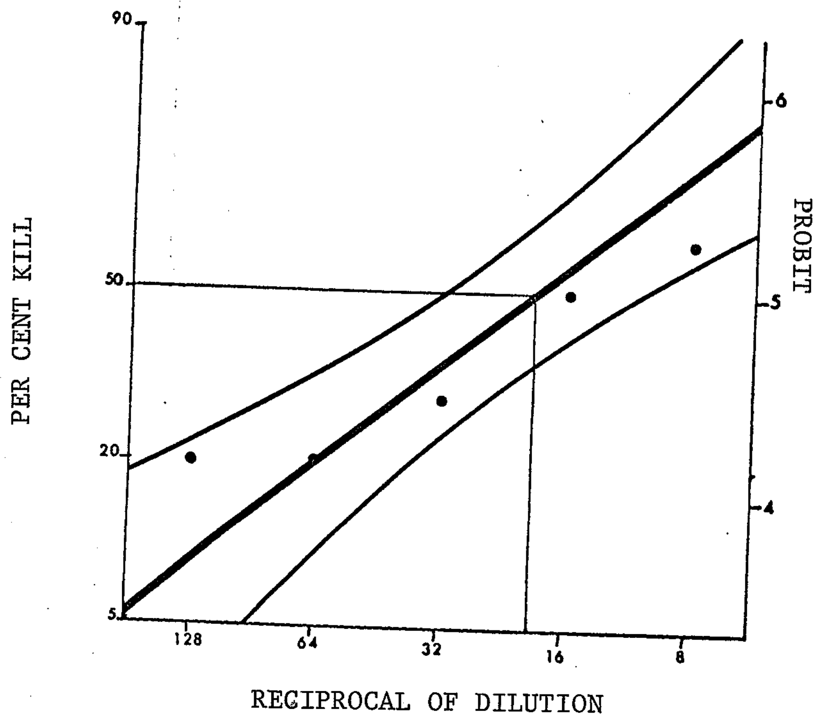
Estimation of ED50 dilution of C37 - strain as a preliminary step for deriving the LD50 in plaque counts.

C37 - Experiment 1



ED50 dilution = 0.0140
Estimated count of plaques in 0.5 ml
of undiluted C37 suspension = K = 114.662
LD50 = 0.0140 x 114.662 = 1.615

C37 - Experiment 2



ED50 dilution = 0.0522
Estimated count of plaques in 0.5 ml
of undiluted C37 suspension = K = 33.548
LD50 = 0.0522 x 33.548 = 1.75

FIGURE 17.

C37 — EXPERIMENT 1

RANKITS: 3, 2, 1, 0, −1, −2

LOG DAYS: 1.00, 1.02, 1.04, 1.06, 1.08, 1.10, 1.12, 1.14, 1.16

DIL1 ——— ×
DIL2 – – – – ●
DIL3 —— —— ○
DIL4 - - - - - □
DIL5 —·—·— ▼

C37 — EXPERIMENT 2

RANKITS: 3, 2, 1, 0, −1, −2, −3

LOG DAYS: 1.00, 1.02, 1.04, 1.06, 1.08, 1.10, 1.12, 1.14, 1.16, 1.18

DIL1 ——— ×
DIL2 – – – – ●
DIL3 —— —— ○
DIL4 - - - - - □
DIL5 —·—·— ▼
DIL6 ··········· ■

... days by Rankit Transformation

There were no deaths among the control groups of mice in all the above experiments during the twenty days' observation period.

Details of the RT50 values of various dilutions employed in respect of each of the five strains of toxoplasma in two experiments are given separately in table 21. Values of RT50 of the strains in each experiment are given as the geometric mean expressed in terms of days.

It is known, by rule of thumb, that the more virulent a strain of organism the smaller the LD50 dose and the shorter the RT50. In other words, virulence of an organism is directly related to the reciprocals of LD50 as well as RT50 value. For each strain of toxoplasma there were three values, namely, reciprocal of LD50, reciprocal of RT50, and product of reciprocals of LD50 and RT50, with which to examine the relative virulence independently and in combination and to compare with the corresponding values for the other strains. For the purpose of comparison each of the three values was normalized, taking the highest of the ten values for the five strains in respect of each of LD50, RT50, and their product at 100 as outlined in table 22.

The null hypothesis that all strains of toxoplasma have the same virulence was rejected by an analysis of variance of LD50* values in two experiments for each strain at $\alpha = 0.10$, of RT50* at $\alpha = 0.01$ and of normalized virulence* (normalized product of reciprocals of LD50 and RT50) at $\alpha = 0.05$.

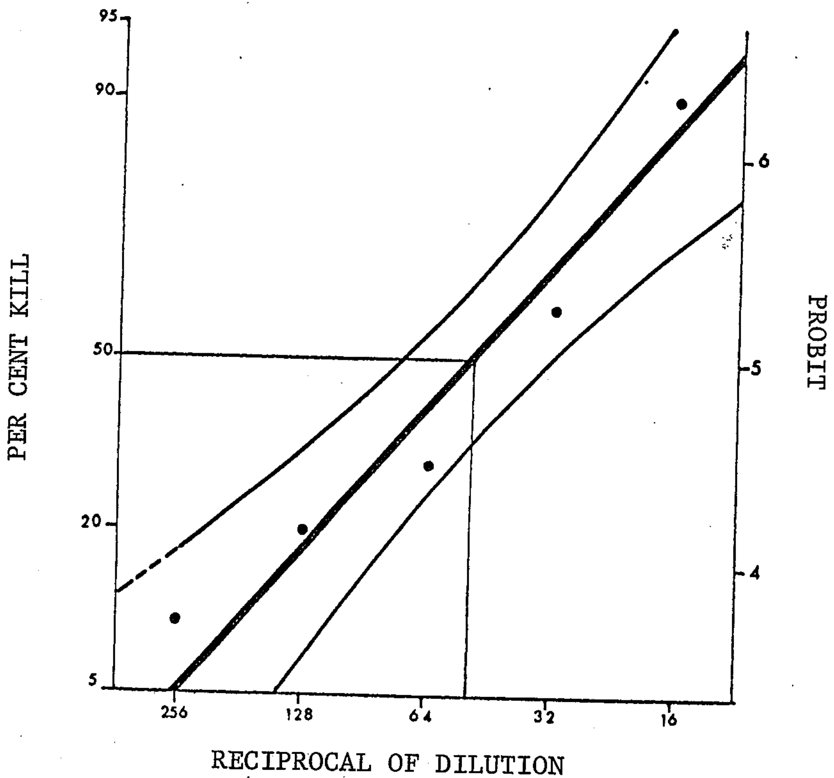
Individual comparisons were made using the Least Significant Difference method (LSD) as given in table 23, significant differences at $\alpha = 0.05$

* In the analyses the logarithmic transformation was used.

FIGURE 18

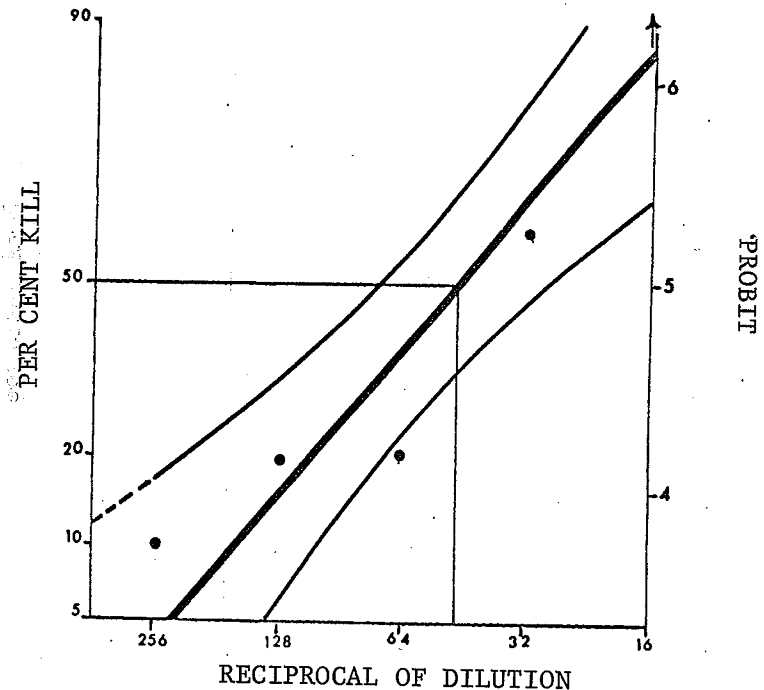
Estimation of ED50 dilution of Beverley - strain as a preliminary step for deriving the LD50 in plaque counts.

Bevérley - Experiment 1



ED50 dilution = 0.0202
Estimated count of plaques in 0.5 ml of undiluted Beverley suspension = K = 47.794
LD50 = 0.0202 x 47.794 = 0.966

Beverley - Experiment 2



ED50 dilution = 0.0209
Estimated count of plaques in 0.5 ml of undiluted Beverley suspension = K = 110.452
LD50 = 0.0209 x 110.452 = 2.314

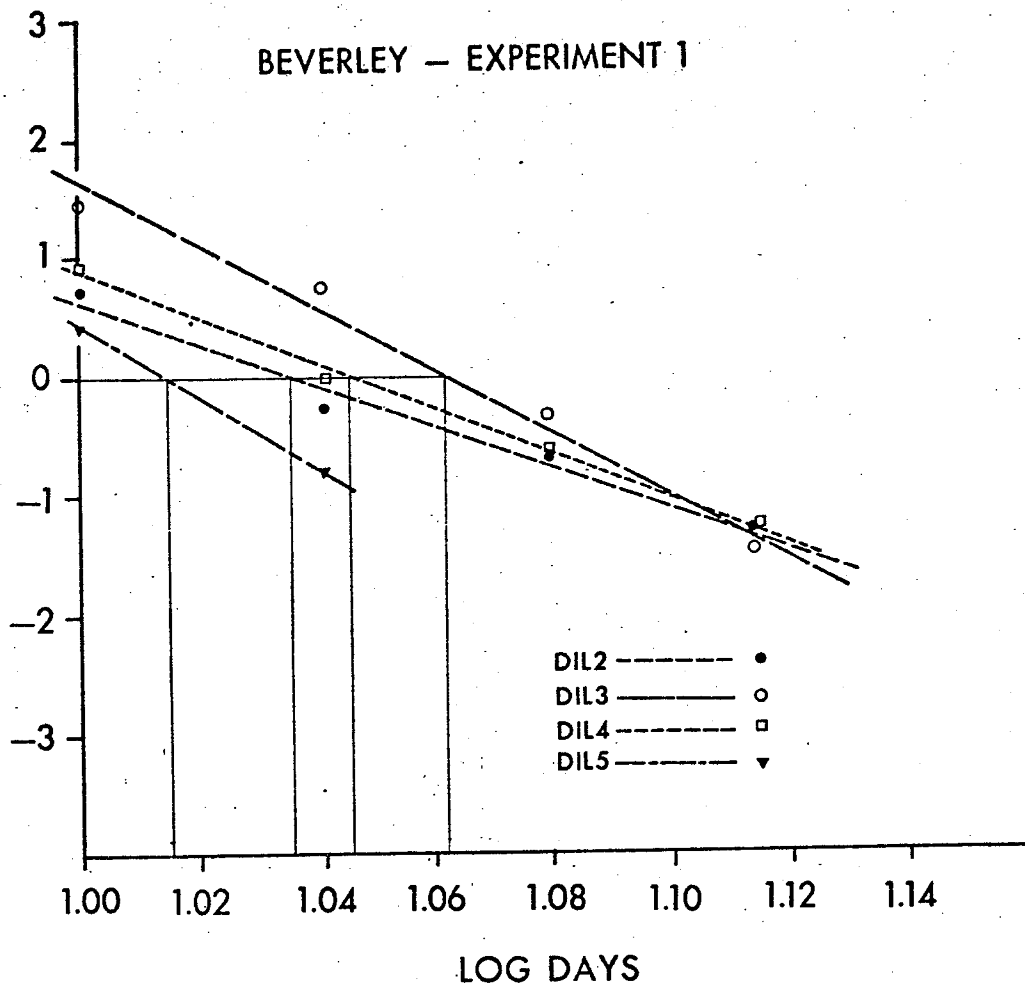


FIGURE 19.

Figure 19: Estimation of RT50 of Beverley strain in terms of days by Rankit Transformation.

TABLE 21 PARTICULAR OF RT50 OF VARIOUS DILUTIONS EMPLOYED IN THE TWO EXPERIMENTS FOR EACH STRAIN OF TOXOPLASMA

STRAIN OF TOXOPLASMA	EXPERIMENT I			EXPERIMENT 2		
	Dilution Number	RT50 of each Dilution in Log Days	RT50 of Experiment I	Dilution Number	RT50 of each Dilution in Log Days	RT50 of Experiment 2
RH	1	0.952	Antilog 0.961 = 9.140 days	2	0.938	Antilog 0.953 = 8.975 days
	2	0.967		3	0.941	
	3	0.961		4	0.954	
	4	0.954		5	0.977	
	5	0.970		6	0.954	
V16	D	0.988	Antilog 1.023 = 10.54 days	2	0.977	Antilog 1.022 = 10.52 days
	1	1.008		3	1.013	
	2	1.014		4	1.029	
	3	1.049		5	1.040	
	4	1.034		6	1.020	
	5	1.047		7	1.020	
	6	1.020		8	1.054	
C56	2	1.032	Antilog 1.046 = 11.12 days	1	1.007	Antilog 1.014 = 10.33 days
	3	1.028		2	1.005	
	4	1.046		3	1.011	
	5	1.054		4	1.020	
	6	1.060		5	1.041	
	7	1.060		6	1.000	
	8	1.041				
C37	1	1.033	Antilog 1.108 = 12.82 days	1	1.056	Antilog 1.078 = 11.97 days
	2	1.096		2	1.108	
	3	1.078		3	1.085	
	4	1.100		4	1.066	
	5	1.097		5	1.060	
	6	1.176		6	1.095	
	7	1.176				
Beverley	2	1.038	Antilog 1.069 = 11.66 days	2	1.116	Antilog 1.113 = 12.97 days
	3	1.063		3	1.114	
	4	1.046		4	1.111	
	5	1.014		5	1.111	
	6	1.079		6	1.113	
	7	1.176		7	1.113	

TABLE 22

DETAILS OF DERIVATION OF NORMALIZED VIRULENCE DERIVED FROM RECIPROCALS OF LD50, RT50, AND THEIR PRODUCT, FOR THE FIVE STRAINS OF TOXOPLASMA IN TWO SEPARATE EXPERIMENTS

Strain of Toxo-plasma	Experiment Number	LD50	Reciprocal of LD50 $\left(\frac{1}{LD50}\right)$	Normalized Virulence Derived from LD50	RT50	Reciprocal of RT50 $\left(\frac{1}{RT50}\right)$	Normalized Virulence Derived from RT50	Product of Reciprocals of LD50 & RT50 $\left(\frac{1}{LD50}\right) \times \left(\frac{1}{RT50}\right)$	Normalized Virulence Derived from Product of Reciprocals of LD50 and RT50
RH	1	.369 ± .074*	$\frac{1}{.369}$	100	9.140	$\frac{1}{9.140}$	98	0.2965	100
	2	.671 ± .153	$\frac{1}{.671}$	55	8.975	$\frac{1}{8.975}$	100	0.1661	56
V16	1	.697 ± .151	$\frac{1}{.697}$	53	10.54	$\frac{1}{10.54}$	85	0.1361	46
	2	.526 ± .096	$\frac{1}{.526}$	70	10.52	$\frac{1}{10.52}$	85	0.1807	61
C56	1	.924 ± .164	$\frac{1}{.924}$	40	11.12	$\frac{1}{11.12}$	81	0.0973	33
	2	.664 ± .199	$\frac{1}{.664}$	56	10.33	$\frac{1}{10.33}$	87	0.1458	49
C37	1	1.615± .295	$\frac{1}{1.615}$	23	12.82	$\frac{1}{12.82}$	70	0.0483	16
	2	1.750± .331	$\frac{1}{1.750}$	21	11.97	$\frac{1}{11.97}$	75	0.0477	16
BEV	1	0.966± .198	$\frac{1}{0.966}$	38	11.66	$\frac{1}{11.66}$	77	0.0888	30
	2	2.314± .507	$\frac{1}{2.314}$	16	12.97	$\frac{1}{12.97}$	69	0.0333	11

* Standard error = $\sqrt{K^2 . var(ED50) + (ED50)^2 . varK}$.

being observed between the following in respect of the LD50 values: RH & C37, RH & Beverley and V16 & C37, but not between RH & V16, RH & C56, V16 & C56, V16 & Beverley* C56 & C37, C56 & Beverley, and C37 & Beverley.

Comparisons of RT50 values showed significant differences between all strains except V16 & C56 and C37 & Beverley.

Similarly, comparisons of the normalized virulences gave significant differences between all strains except RH & V16, RH & C56, V16 & C56, C56 & C37, C56 & Beverley, and C37 & Beverley.

Finally, using all the three values, namely, LD50, RT50, and normalized virulence, it appears to be possible to differentiate all the strains from one another except V16 from C56, and C37 from Beverley.

* Very close to significance.

TABLE 23

MULTIPLE COMPARISON OF DIFFERENT STRAINS OF TOXOPLASMA BY LSD METHOD FOR LD50, RT50, AND NORMALIZED VIRULENCE+ AT $\alpha = 0.05$

Toxoplasma Strains Compared	LD50	RT50	Normalized Virulence+
RH : V16	NS	Sign	NS
RH : C56	NS	Sign	NS
RH : Beverley	Sign	Sign	Sign
RH : C37	Sign	Sign	Sign
V16: C56	NS	NS	NS
V16: C37	Sign	Sign	Sign
V16: Beverley	NS*	Sign	Sign
C56: C37	NS	Sign	NS
C56: Beverley	NS	Sign	NS
Bev: C37	NS	NS	NS

* Very close to significance

NS = Not Significant

Sign = Significant

+ Normalized Virulence as derived from the product of reciprocals of LD50 and RT50.

IMMUNODIFFUSION AND IMMUNOELECTROPHORESIS EXPERIMENTS

Experiment 1

Immunodiffusion study of different preparations of toxoplasma(RH) and (V16) antigens.

Filling three antigenic preparations of RH strain from peritoneal exudate of mice, namely, sediment, supernatant and distilled water (DW) lysed antigens in different peripheral wells and rabbit antiRH serum in the central well, diffusion was allowed to take place for 48 hours as detailed under materials and methods. Similarly, sediment, supernatant and DW lysed antigenic preparations of V16 strain was allowed to react with rabbit antiV16 serum.

As can be seen in figure 20 DW lysed antigens of RH and V16 did not give any reaction. RH sediment and supernatant gave identical lines of precipitation. In the case of V16 strain, only the supernatant gave three distinct precipitin lines.

Experiment 2

Immunodiffusion study of different preparations of toxoplasma(RH) antigen and chicken antiRH serum.

When the experiment was carried out on similar lines as above using chicken antiRH serum in place of rabbit antiRH serum, no precipitin lines developed, even as the reactants were allowed to diffuse up to 7 days.

Supernatant preparations have been reported to yield maximum number of lines of precipitation as compared to sediment and DW lysed antigens, the latter as being poorly reactive. As supernatant antigenic preparations of both RH and V16 strains were found to give maximum number of pre-

cipitin bands, it was preferred to sediment and DW lysed antigens and was employed in the experiments hereafter.

Experiment 3.

Study of immunodiffusion with five strain specific rabbit anti-toxoplasma sera and five strains of toxoplasma antigens.

In a preliminary immunodiffusion study using serial two-fold dilutions of supernatant antigens in physiological saline from undiluted to 1-64 dilutions, the dilutions of antigen giving the maximum precipitin lines in respect of each strain against its homologous and heterologous antisera was determined and employed in this study. Each strain specific rabbit antiserum was allowed to react for 48 hours with five different strain supernatant antigens in different peripheral wells. As controls, when rabbit preimmune serum and physiological saline were allowed to diffuse separately against the five strain specific supernatants, no precipitin lines developed.

From the results shown in figure 21, it can be seen that all the five strains of toxoplasma share a common group antigenic determinant which is seen as a thick, diffuse band, sometimes seen to comprise 3 to 4 lines. An additional precipitin line was appreciable with V16 and Beverley and possibly C56 antigens in their reaction with antiV16 serum. Similarly, an additional line was apparent with V16 and Beverley antigens on diffusion against antiBeverley serum.

When the supernatant antigens were prepared again and the experiments repeated, similar results were obtained.

Experiment 4

Demonstration of mouse protein(s) in antigenic materials and antibody to mouse protein(s) in rabbit antitoxoplasma sera.

Rabbit antimouse serum was allowed to react with RH -sediment, -supernatant, and -DW lysed antigens for 24 hours on agarose slides (figure 22A). Sediment and supernatant antigens gave 3 distinct lines of precipitation. DW lysed antigen did not react. Similarly, rabbit antimouse serum was allowed to diffuse against supernatant antigens of individual strains of toxoplasma to demonstrate that the antigenic preparations from the mouse exudate contained mouse protein(s) as contaminant (figure 22B). All the five supernatant antigens and NMS gave a thick, diffuse band and a distinct line of precipitation.

When each strain specific rabbit antitoxoplasma serum was tested against NMS* as shown in figuree 22C, multiple precipitin bands were seen indicating presence of antibodies to mouse protein(s) in the antisera. Normal rabbit preimmune serum used as a reference did not react with NMS.

Experiment 5.

Demonstration of antibodies to toxoplasma in rabbit antitoxoplasma sera as distinct from the antibodies to mouse protein(s).

Rabbit antiRH serum was taken in the central well and the five supernatant antigens and NMS were allowed to diffuse from the peripheral wells. Similarly, antiRH and antiC56 sera were separately allowed to diffuse against the five supernatant antigens and normal mouse peritoneal exudate (NME)* (figure 23A, B, and C). Whereas no additional lines of pre-

* NMS contained about 70 mg protein/ml. It was diluted 1-12 in physiological saline and used in the experiments.

* NME employed in the experiments contained 10.5 mg protein/ml.

FIGURE 20

Immunodiffusion study of different preparations of Toxoplasma RH and V16 antigens.

	Central Well	Peripheral Well
A.	Rabbit antiRH serum	1. RH sediment
		2. "
		3. RH supernatant
		4. "
		5. RH DW lysed antigen
		6. "
B.	Rabbit antiV16 serum	1. V16 sediment
		2. "
		3. V16 supernatant
		4. "
		5. V16 DW lysed antigen
		6. "

FIGURE 21

Study of immunodiffusion with five strain specific rabbit antitoxoplasma sera and five strains of toxoplasma antigens.

	CW	PW LEFT	PW RIGHT
A.	Rabbit antiRH serum	Appropriate dilutions of:	Further two-fold dilutions of:
		1. RH supernatant	1. RH supernatant
		2. V16 "	2. C56 "
		3. C56 "	3. V16 "
		4. C37 "	4. C56 "
		5. Beverley "	5. Beverley "
		6. V16 "	6. C37 "
B.	Rabbit antiV16 serum	As in A	As in A
C.	Rabbit antiC56 serum	As in A	As in A
D.	Rabbit antiC37 serum	As in A	As in A
E.	Rabbit antiBeverley serum	As in A	As in A

Figure 20.

A

B

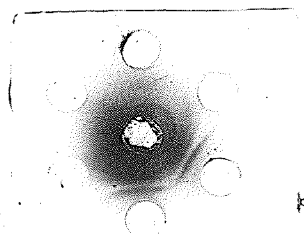
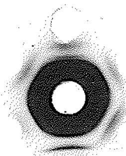
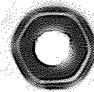


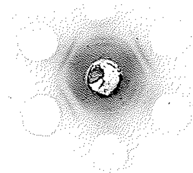
Figure 21.

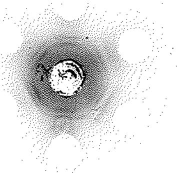
A



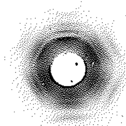


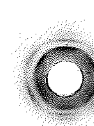
B



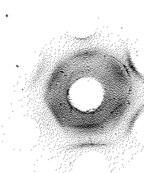


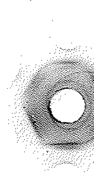
C





D





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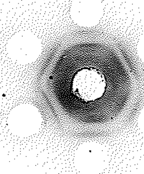
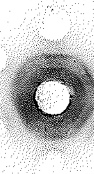




cipitin was evident with the various supernatant strain antigens as compared to NMS in their reaction with antiRH serum (vide figure 23A), additional lines of precipitation were manifest with all the five antigens as compared to NME while reacting with both antiRH and antiC56 sera (vide figures 23B and C).

The results reveal that NMS contains besides the determinants contained in NME some additional determinants which seem to react with the antitoxoplasma sera the same way as the supernatant antigens.

Experiment 6.

Immunodiffusion study after absorption of rabbit antiRH serum with normal mouse serum dry powder.

In order to study the precipitating antibodies to toxoplasma only in the rabbit antitoxoplasma serum*, an attempt was made to absorb the antibodies to mouse protein(s) with NMS dry powder in the ratio of 3:1, 2:1, 1:1, and 1:2 and the absorbed serum was allowed to react in agarose gel with five different strain supernatant antigens, with NMS as control (figure 24A and B).

Similarly, other strain specific antitoxoplasma sera were separately absorbed with NMS dry powder in the ratio of 1:1 and 1:2 and studied as above (figure 24C and D).

Absorption of antiRH serum with increased amount of NMS dry powder resulted in gradual disappearance of precipitin bands. V16 and Beverley antigens gave 1 or 2 precipitin lines and NMS gave 1 line after absorption of antiRH at 1:1 ratio but the lines disappeared after absorption at 1:2 ratio.

* Rabbit antiRH serum.

FIGURE 22

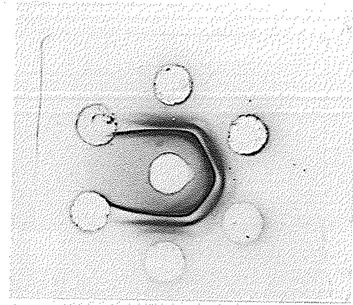
Demonstration of mouse protein(s) in antigenic materials and antibody to mouse protein(s) in rabbit antitoxoplasma sera.

	CW		PW
A.	Rabbit antimouse serum	1.	RH sediment
		2.	"
		3.	RH supernatant
		4.	"
		5.	RH DW lysed antigen
		6.	"
B.	Rabbit antimouse serum	1.	RH supernatant
		2.	V16 supernatant
		3.	C56 "
		4.	C37 "
		5.	Beverley "
		6.	Normal mouse serum
C.	Normal mouse serum	1.	Rabbit antiRH serum
		2.	Rabbit antiV16 "
		3.	" antiC56 "
		4.	" antiC37 "
		5.	" antiBeverley serum
		6.	Rabbit preimmune serum

FIGURE 23

Demonstration of antibodies to toxoplasma in rabbit antitoxoplasma sera as distinct from the antibodies to mouse protein(s).

	CW		PW LEFT		PW RIGHT
A.	R antiRH serum	1.	RH supernatant	1.	Beverley supernatant
		2.	NMS	2.	NMS
		3.	V16 supernatant	3.	C37 supernatant
		4.	C56	4.	V16 "
		5.	NMS	5.	NMS
		6.	C 37 supernatant	6.	C56 supernatant
B.	R antiRH serum	1.	RH supernatant	1.	Beverley supernatant
		2.	NME	2.	NME
		3.	V16 supernatant	3.	C37 supernatant
		4.	C56 supernatant	4.	V16 "
		5.	NME	5.	NME
		6.	C37 supernatant	6.	C56 supernatant
C.	R antiC56 serum		As in B		As in B



A

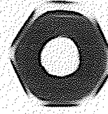
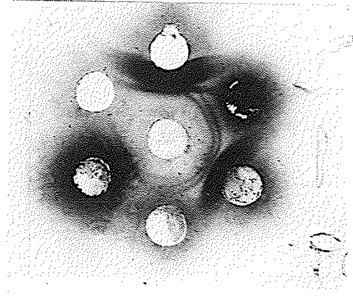


Figure 22. B



C

A

Figure 23. B

C

FIGURE 24

Immunodiffusion study after absorption of rabbit antiRH serum with normal mouse serum dry powder.

	LEFT		RIGHT	
	CW	PW	CW	PW
A.	R antiRH absorbed with NMS 3:1	1. RH antigen 2. V16 " 3. C56 " 4. C37 " 5. Beverley 6. NMS	R antiRH absorbed with NMS 2:1	As in PW A.
B.	R antiRH absorbed with NMS 1:1	As in PW A	R antiRH absorbed with NMS 1:2	As in PW A.
C.	R antiV16 absorbed with NMS 1:1	1. C37 antigen 2. Beverley 3. NMS 4. RH antigen 5. V16 " 6. C56 "	R antiC56 absorbed with NMS 1:1	1. C37 antigen 2. Beverley 3. NMS 4. RH antigen 5. V16 " 6. C56 "
D.	R antiC37 absorbed with NMS 1:1	As in PW A	R antiBeverley absorbed with NMS 1:1	As in PW A.

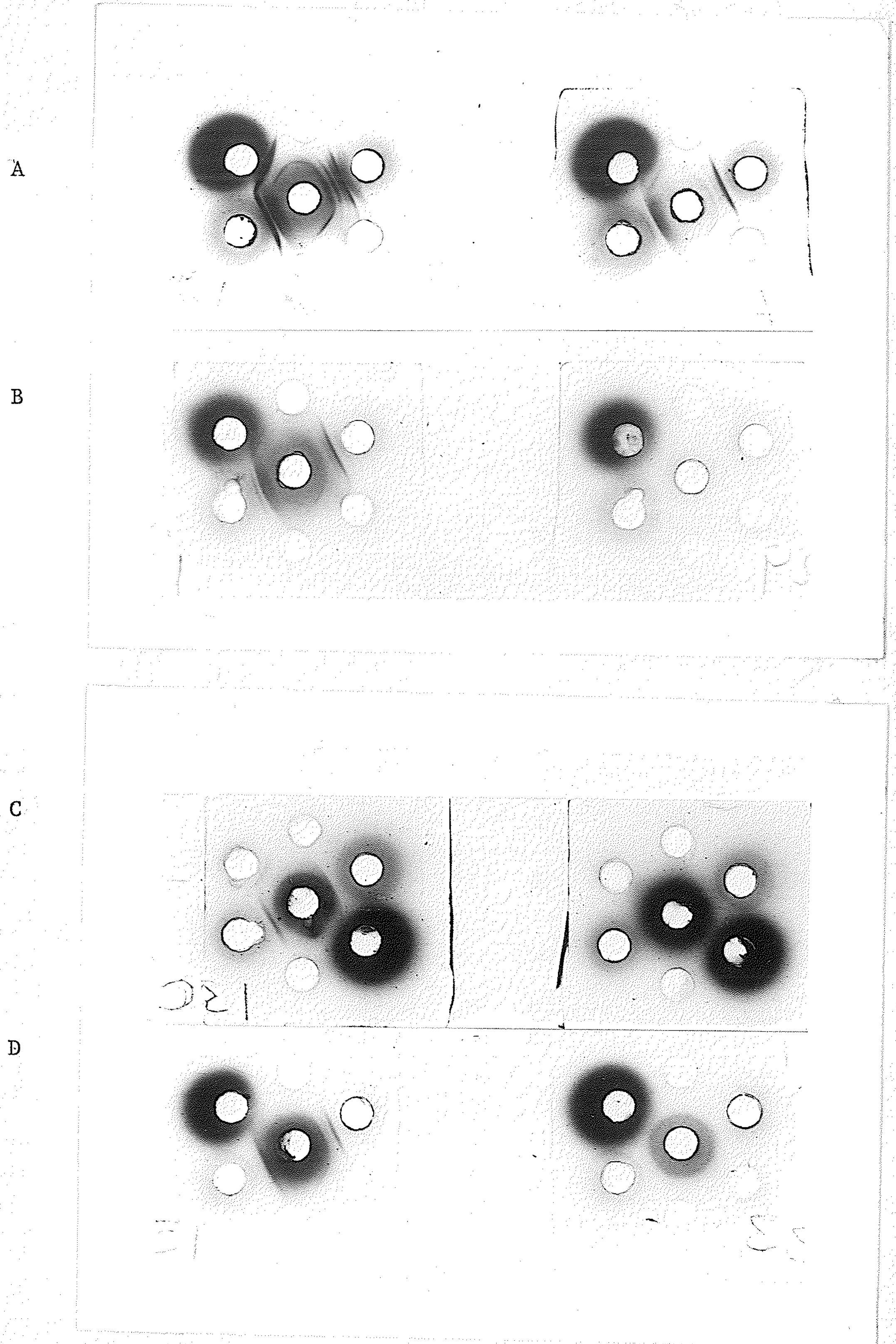


Figure 24.

Similarly, other strain specific antitoxoplasma sera, after absorption with NMS at 1:2 ratio did not react with any antigens. However, at 1:1 ratio of absorption of antiV16 serum, V16 and Beverley gave one distinct line of precipitation and NMS seemed to give one faint precipitin line. AntiC37 serum, after absorption at 1:1 ratio gave 1 line of precipitation with V16 and Beverley antigens.

Experiment 7.

Immunodiffusion study after absorption of rabbit antitoxoplasma sera with NMS-immunosorbent.

Precipitin lines obtained on diffusion of NMS-immunosorbent-absorbed strain specific antitoxoplasma sera against five strains of toxoplasma supernatants and NMS separately are given in figure 25.

Each strain specific antitoxoplasma serum, after absorption seemed to react with V16, Beverley and NMS but not with RH, C56 and C37 antigens.

Experiment 8.

Immunodiffusion study after absorption of rabbit antiRH serum with different toxoplasma strains.

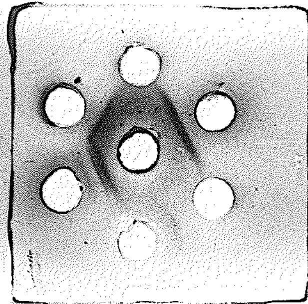
Investigating into the possibility of studying the strain specific antigens not shared by other strains, the following experiments were carried out. Rabbit antiRH serum absorbed separately with an equal volume and with twice its volume of different strains of toxoplasma pellet as given in materials and methods was allowed to react with different strain supernatant antigens and NMS.

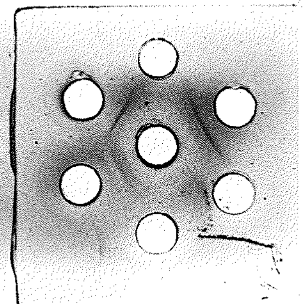
FIGURE 25

Immunodiffusion study after absorption of rabbit antitoxoplasma sera with NMS-immunosorbent.

	LEFT		RIGHT	
	CW	PW	CW	PW
A.	R antiRH absorbed with NMS-immuno-sorbent	1. RH antigen 2. V16 " 3. C56 " 4. C37 " 5. Bev. " 6. NMS	R antiV16 absorbed with NMS-immuno-sorbent	As in PW A
B.	R antiC56 absorbed with NMS-immuno-sorbent	As in PW A	R antiC37 absorbed with NMS-immuno-sorbent	As in PW A
C.	R antiBev absorbed with NMS-immuno-sorbent	As in PW A		

A





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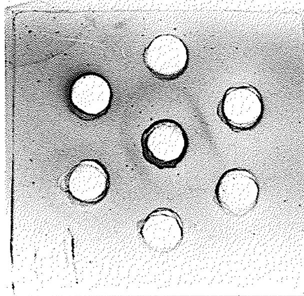

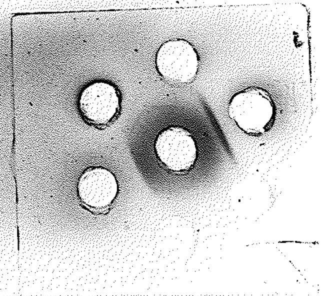

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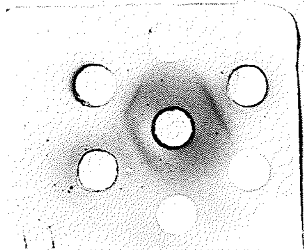


Figure 25.

AntiRH serum absorbed with an equal volume of Beverley strain reacted with V16, Beverley and NMS, showing in each case, one line of precipitation (figure 26). Absorption at 1:2 with Beverley rendered the serum non-reactive. There was no reaction when antiRH serum was absorbed separately with V16, C56 and C37 at 1:1 as well as at 1:2 ratio.

Experiment 9.

Absorption of rabbit antiV16 serum with graded amounts of RH strain toxoplasma and diffusion against different strain antigens.

After absorption of rabbit antiV16 serum with RH toxoplasma pellet in the ratio of 4:1, 3:1, 2:1, and 1:1, it was allowed to react separately with the five strain supernatants and the results obtained are shown in figure 27.

Whereas V16 and possibly Beverley, each gave a precipitin line with antiV16 serum absorbed at 4:1, 3:1, and 2:1 ratio, there was no reaction with antiV16 absorbed with RH at 1:1 ratio. When the absorbed serum was allowed to react with NMS, one precipitin line was seen with antiV16 absorbed at 4:1 and 3:1 but not with serum absorbed at 2:1 and 1:1 ratios.

The results of absorption experiments seem to indicate that either there is non-specific absorption of antibodies to toxoplasma by NMS as well as non-specific absorption of toxoplasma antibodies by heterologous strains of toxoplasma when the absorption is carried out at a greater ratio or that there are no precipitating antibodies directed to toxoplasma in the various strain specific antitoxoplasma sera as distinct from antibodies directed to mouse-protein(s) contaminant. To ascertain if precipitating antibodies directed to toxoplasma antigens are actually present in antitoxoplasma sera, the following experiments were carried out.

Experiment 10.

Immunodiffusion study of tissue culture toxoplasma strain antigens and rabbit antitoxoplasma sera.

Each strain specific antitoxoplasma serum was allowed to diffuse separately for 48 hours against the five strains of toxoplasma antigens from tissue cultures (TC) in peripheral wells. The results of immunodiffusion can be seen in figure 28. A diffuse precipitin band was seen common to all the five strains of toxoplasma with antiRH and antiV16 sera. The reaction was not distinct with the other three antisera and hence not shown here.

When rabbit antimouse serum was allowed to diffuse against five strains of toxoplasma TC antigens*, no precipitin lines developed, indicating that the TC antigenic materials were free from mouse protein(s). Further the growth medium used in TC or the calf serum which was a component of the growth medium when allowed to react with antiRH and antiV16 sera separately, no reaction was observed. Calf serum was tested undiluted as well as in two-fold dilutions up to 1-32 dilution. Normal rabbit preimmune serum also did not react with TC antigens. Control TC antigen prepared by inoculating Vero monolayers with mouse peritoneal exudate which was treated similarly as the toxoplasma from the mouse exudate used for inoculation of TC's for the preparation of TC toxoplasma antigen, and the fluid collected and treated the same way as in the preparation of TC toxoplasma antigens as outlined in materials and methods, when allowed to diffuse against antiRH or antiV16 sera, showed no reaction.

* TC antigens contained about 30 mg protein/ml.

FIGURE 26

Immunodiffusion study after absorption of rabbit antiRH serum with different toxoplasma strains.

CW	PW
R antiRH absorbed with Beverley 1:1	1. RH antigen 2. V16 " 3. C56 " 4. C37 " 5. Bev " 6. NMS

FIGURE 27

Absorption of rabbit antiV16 serum with graded amounts of RH strain toxoplasma and diffusion against different strain antigens.

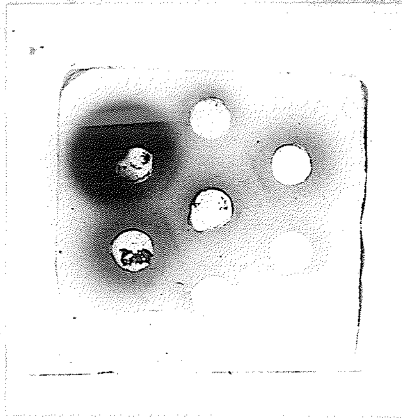
	LEFT		RIGHT	
	CW	PW	CW	PW
A.	R antiV16 absorbed with RH 4:1	1. RH antigen 2. V16 " 3. C56 " 4. C37 " 5. Bev " 6. saline	R antiV16 absorbed with RH 3:1	As in PW A
B.	R antiV16 absorbed with RH 2:1	As in PW A	R antiV16 absorbed with RH 1:1	As in PW A

FIGURE 28

Immunodiffusion study of tissue culture toxoplasma strain antigens and rabbit antitoxoplasma sera.

LEFT		RIGHT	
CW	PW	CW	PW
R antiRH serum	1. RH TC Ag 2. V16 " 3. C56 " 4. RH " 5. C37 " 6. Bev "	R antiV16 serum	1. V16 TC Ag 2. RH " 3. C56 " 4. V16 " 5. C37 " 6. Bev "

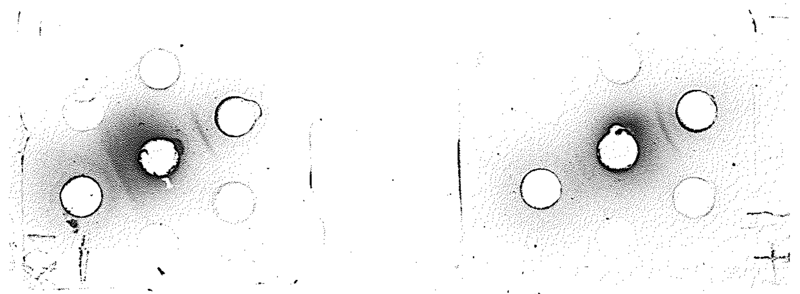
Figure 26.



H

Figure 27.

A



B

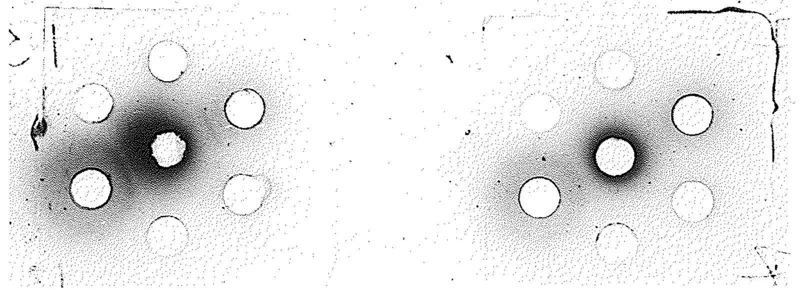
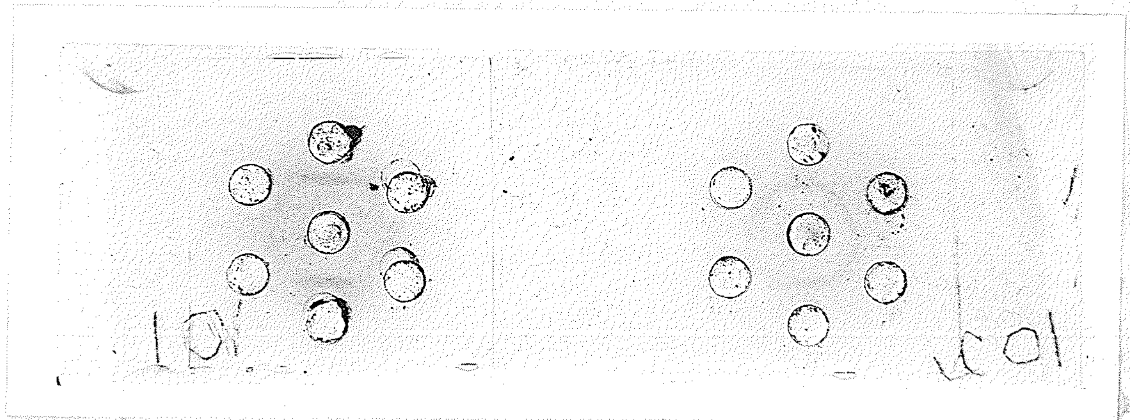


Figure 28.



Experiment 11.

Indirect microradioimmunoassay for detecting antibodies directed to toxoplasma (TC RH) antigen.

The antiRH serum which had been absorbed with lyophilized NMS at 1:2 ratio, and the unabsorbed antiRH serum were tested by indirect micro-RIA for the presence of specific toxoplasma antibodies using TC RH antigen*. Normal rabbit preimmune serum (NRPIS) and rabbit antimouse serum were used as controls. All the sera were separately diluted 1-10 and the TC RH antigen 1-20 with PBS-BSA. Test was carried out as given under materials and methods.

In addition, a similar set of experiment was performed in duplicate without using TC RH antigen (omitting the first step), in order to assess the non-specific adsorption of antisera to the wells.

The results obtained are outlined hereunder.

TEST SYSTEM		RADIOACTIVITY
TC RH Ag + R antiRH absorbed with NMS	+ ^{125}I-labeled Ab.	2462.000 ± 65.510
TC RH Ag + NRPIS	+ ^{125}I-labeled Ab.	2333.250 ± 60.119
TC RH Ag + R antiRH serum	+ ^{125}I-labeled Ab.	3584.281 ± 39.385
TC RH Ag + R antimouse serum	+ ^{125}I-labeled Ab.	2642.750 ± 67.720
R antiRH absorbed with NMS	± ^{125}I-labeled Ab.	1521.000 ± 20.343
NRPIS	+ ^{125}I-labeled Ab.	1362.500 ± 13.257
R antiRH serum	+ ^{125}I-labeled Ab.	1404.500 ± 38.045
R antimouse serum	+ ^{125}I-labeled Ab.	2019.000 ± 89.628

On analysis of the results using t test, it was found that there was no significant difference between antiRH absorbed with lyophilized NMS and NRPIS. The slight increase in radioactivity observed with antiRH

* TC RH antigen contained 7.35 mg protein/ml.

absorbed with NMS compared to NRPIS in the test system is also seen in the same proportion in the blanks in the absence of TC RH antigen.

Highly significant differences were observed between antiRH and NRPIS, antiRH and antiRH absorbed with NMS, as well as between antiRH and antimouse serum ($p < 0.01$) in each case. In the blanks, there was no significant difference between antiRH and NRPIS but significant difference was found between antiRH absorbed with NMS and NRPIS and between NRPIS and antimouse serum.

From the above findings, it may be concluded that antiRH serum contained antibodies directed to toxoplasma (TC RH) and that most of these antibodies, if not all, have been lost on absorption with lyophilized NMS. Further, the higher counts in the blanks with rabbit antimouse serum may be explained by assuming that it contained more immunoglobulins, possibly due to its being a hyper-immune serum produced in rabbits against mouse serum proteins. The evidence obtained does not indicate that TC RH antigen reacts with rabbit antimouse serum any more than TC RH antigen reacts with NRPIS; in other words, TC RH antigen does not seem to have mouse protein(s) as contaminant.

Experiment 12.

Immunoelectrophoretic study of toxoplasma supernatant antigens.

Immunoelectrophoresis (IEP) offers certain advantages over immunodiffusion, in that the different constituents of an antigenic material can be defined on the basis of electrophoretic mobilities. In addition, in IEP, the chances of coalescence of precipitin bands have a very low probability since for coalescence it would require that the two antigenic

determinants would have to have exactly the same electrophoretic mobility, rate of diffusion, and antigen-antibody ratios (Grabar, 1957). Hence, the IEP study.

Each toxoplasma strain supernatant was separated by electrophoresis for 60 minutes on microscope slides containing agarose, and then allowed to diffuse with homologous and heterologous rabbit antitoxoplasma sera. 3 ml agarose/slide was used for the study of antiRH, antiC56 and antiBeverley and 4 ml agarose/slide for antiV16 and antiC37. NME was used as a control against each antiserum (figure 29).

NMS was similarly separated and allowed to diffuse with rabbit anti-mouse serum under the same conditions on separate slides containing 3 ml and 4 ml agarose, and the different bands obtained were arbitrarily divided into γ-β, β , α , and α-albumin zones on paper templates. The bands obtained in each antigen-serum combination as well as NME-serum combination on the original slides were then classified into different groups using the paper templates and the results are shown in table 24. This was done under the belief that the mouse protein(s) contaminant present in the antigen preparations would give bands in the same positions as seen in NME-serum combination, even as it is understood that the number of these bands could be fewer but not more than in the NME-serum combination, depending on the degree of contamination of each antigenic preparation. It therefore follows that the excess number of bands seen in any antigen-serum combination over that seen in NME-serum combination would be due to the antigenic determinants. However, it can be appreciated that bands occupying different positions can come under the same zone*.

* In other words, the precipitin lines found in, say, β region, with different antigens and NME are not necessarily superimposable.

FIGURE 29

Immunoelectrophoretic study of toxoplasma antigens (supernatants)

I.

	Central Trough	Top Well	Bottom Well
A-D	Rabbit antiRH serum	A. RH supernatant	V16 supernatant
		B. C56 supernatant	C37 supernatant
		C. Bev supernatant	RH supernatant
		D. NME	NME

II.

	Central Trough	Top Well	Bottom Well
A-D	Rabbit antiV16 serum	A. RH supernatant	V16 supernatant
		B. C56 supernatant	C37 supernatant
		C. Bev supernatant	RH supernatant
		D. NME	NME

FIGURE 29

Immunoelectrophoretic study of toxoplasma antigens (supernatants)

III.	Central Trough	Top Well	Bottom Well
A-D	Rabbit antiC56 serum	A. RH supernatant	V16 supernatant
		B. C56 supernatant	C37 supernatant
		C. Bev supernatant	RH supernatant
		D. NME	NME

IV.

A-D	Rabbit antiC37 serum	A. RH supernatant	V16 supernatant
		B. C56 supernatant	C 37 supernatant
		C. Bev supernatant	RH supernatant
		D. NME	NME

FIGURE 29

Immunoelectrophoretic study of toxoplasma antigens (supernatants)

V.	Central Trough	Top Well	Bottom Well
A-D	Rabbit antiBeverley serum	A. RH supernatant	V16 supernatant
		B. C56 supernatant	C37 supernatant
		C. Bev supernatant	RH supernatant
		D. NME	NME

Figure 29.

I.

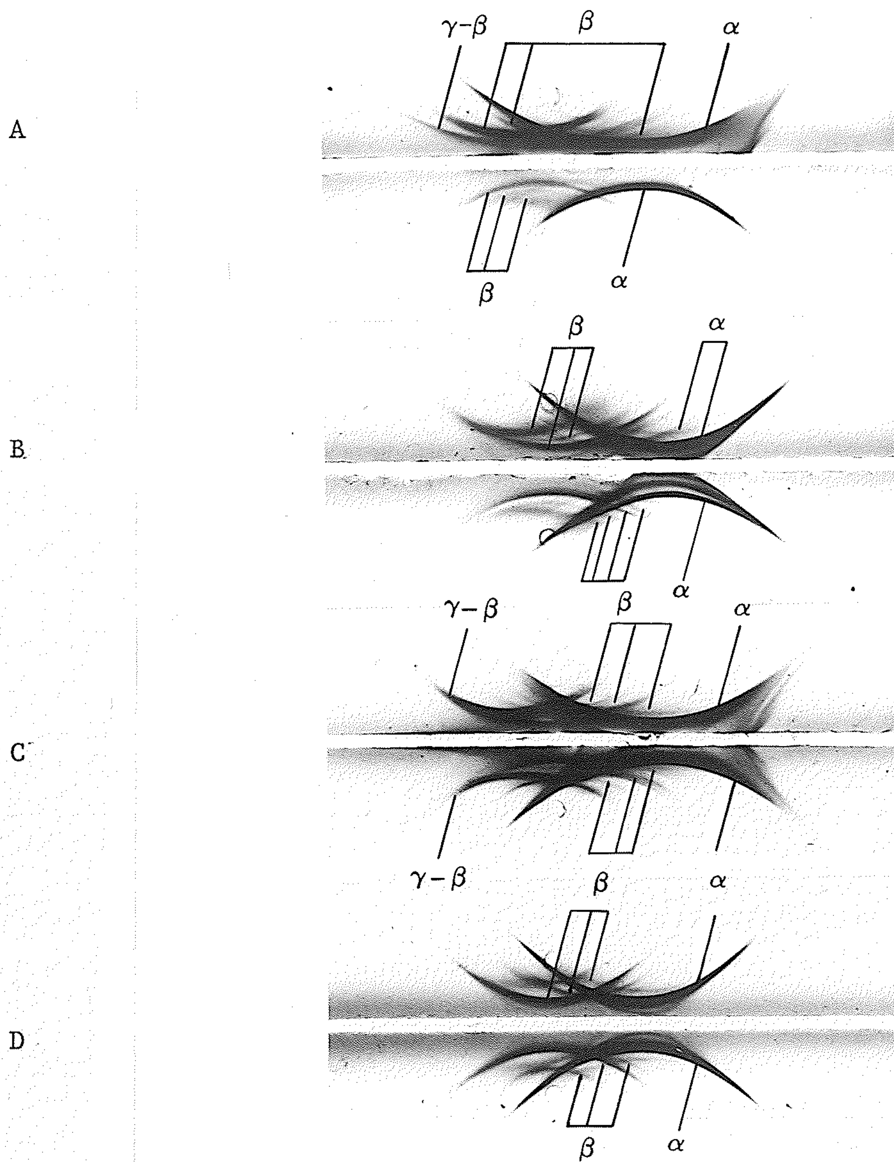


Figure 29.

II.

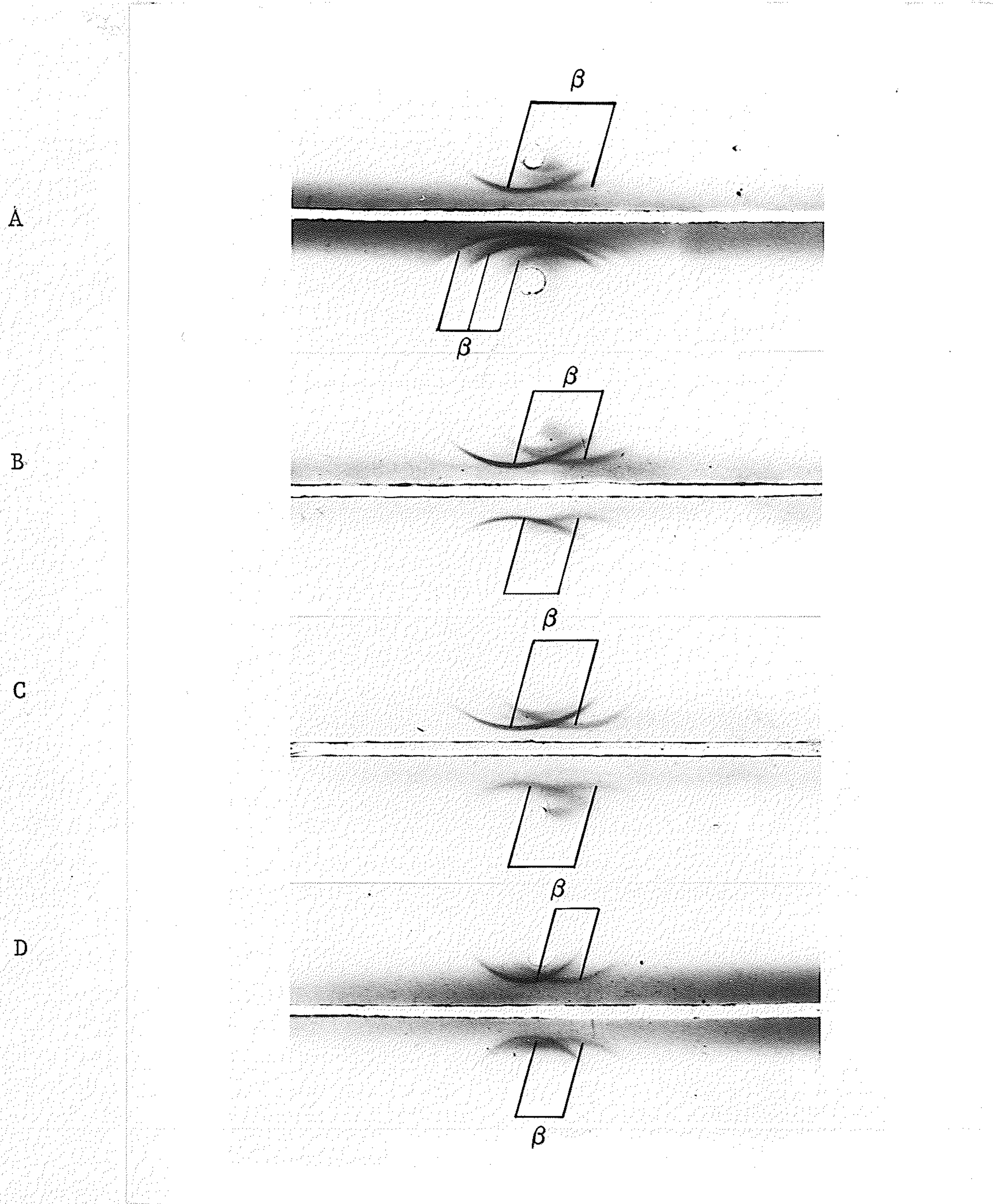


Figure 29.

III.

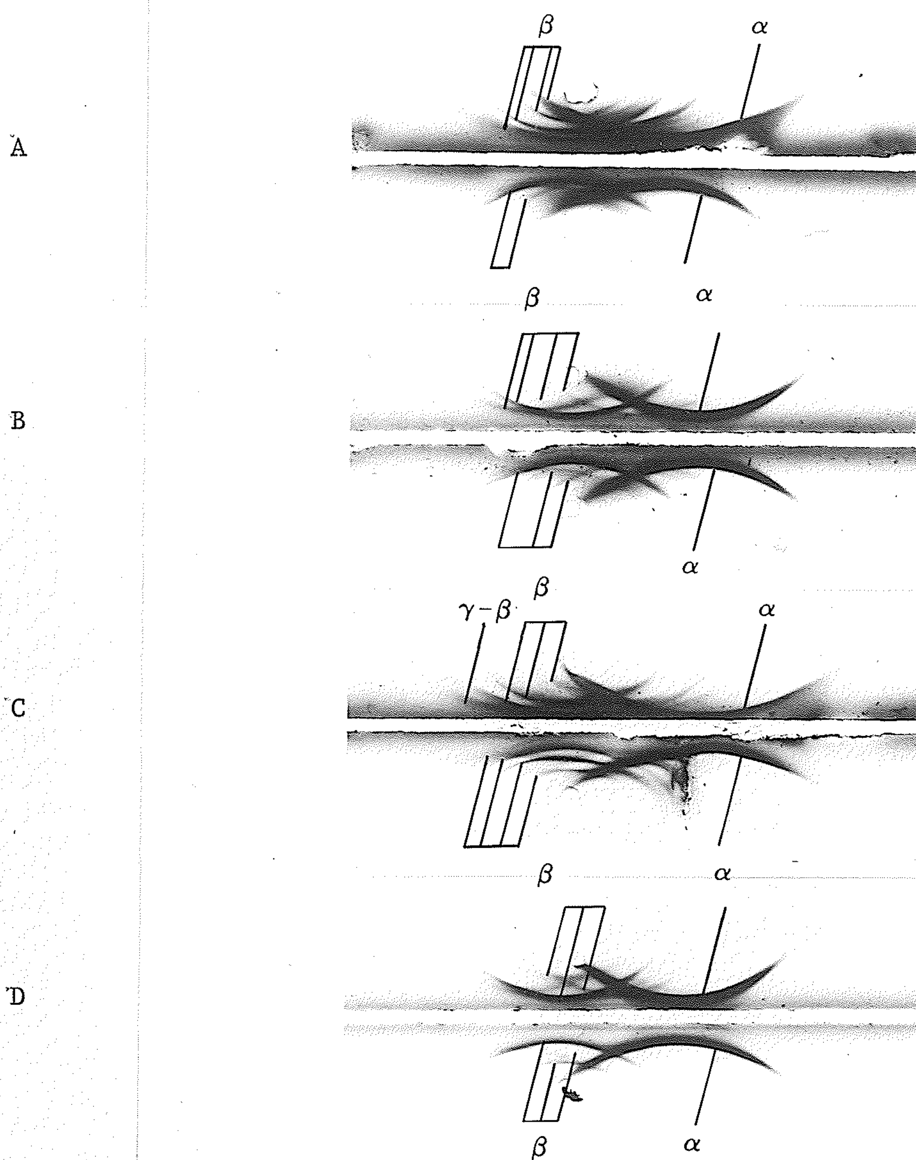
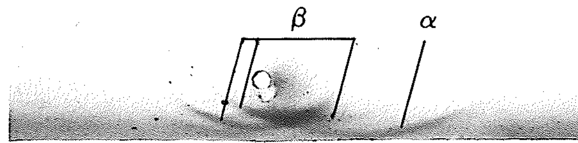


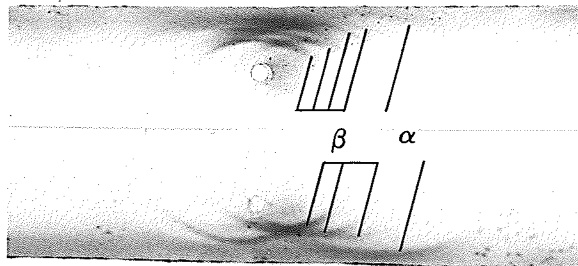
Figure 29.

IV.

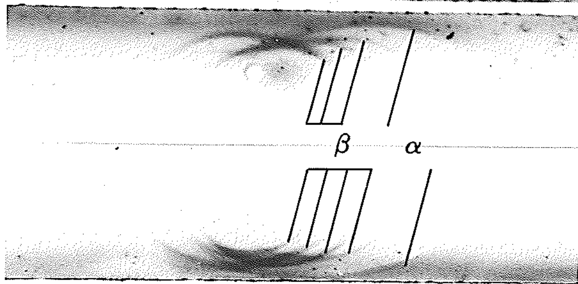
A



B



C



D

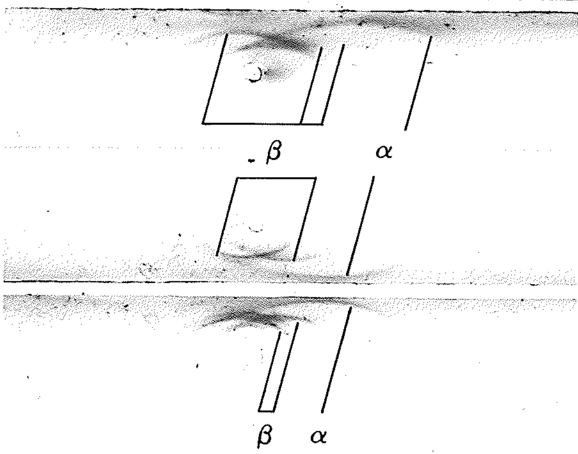


Figure 29.

V.

A

B

C

D

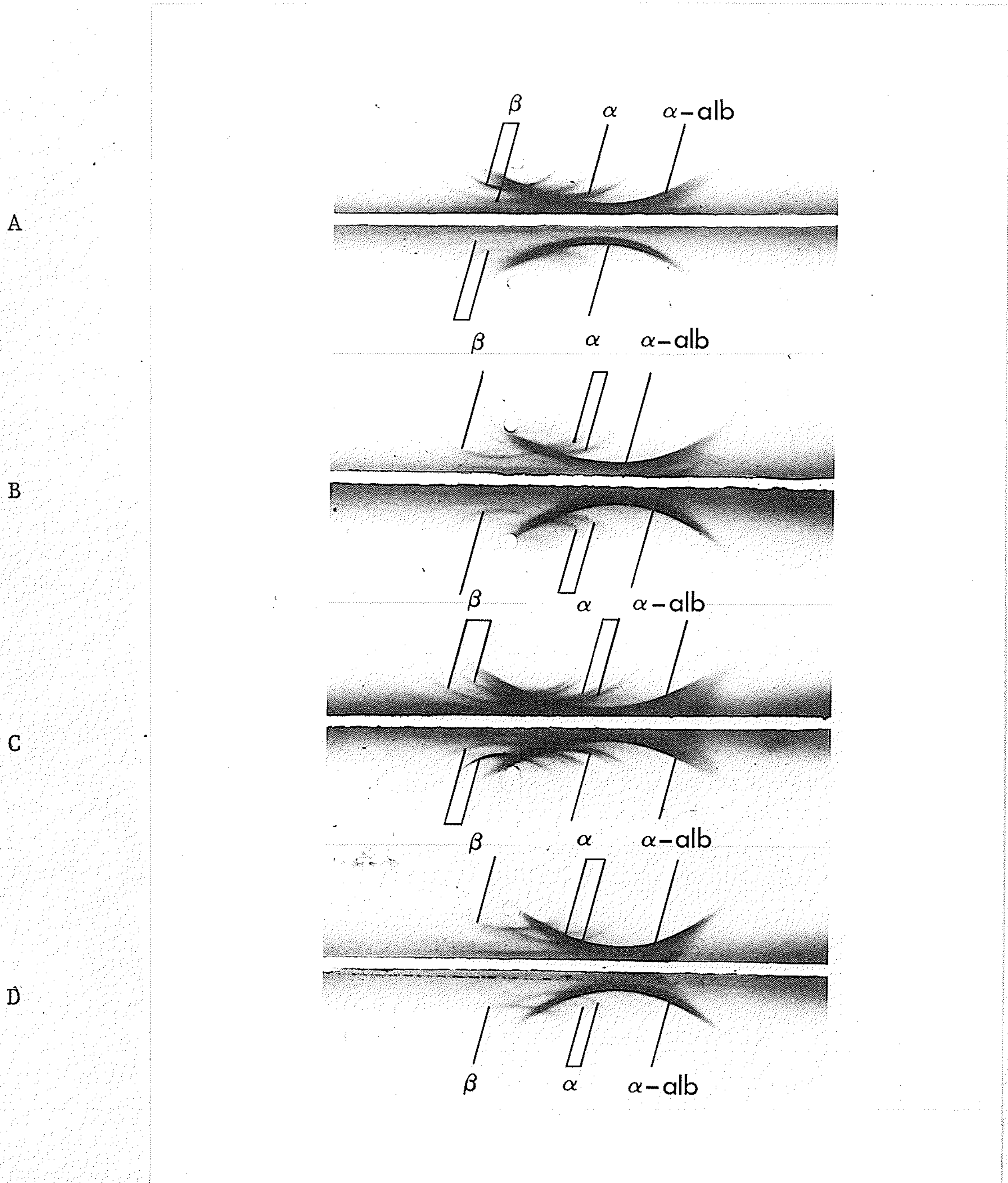


TABLE 24

ARBITRARY CLASSIFICATION OF THE PRECIPITIN BANDS OBTAINED ON IMMUNOELECTROPHORESIS OF FIVE STRAINS OF TOXOPLASMA AND NME WITH EACH OF FIVE STRAIN SPECIFIC ANTISERA WITH AN ATTEMPT TO DETERMINE THE ADDITIONAL BANDS ATTRIBUTABLE TO TOXOPLASMA ANTIGENIC DETERMINANTS

ANTIGENS

	NME (Control)			RH				V16		C56			C37			Beverley			
	β	α	α-alb	γ-β	β	α	α-alb	β	α	β	α	α-alb.	β	α	α-alb.	γ-β	β	α	α-alb.
Anti RH	3	1		1	3	1		3	1	3	2		4	1		1	3	1	
Additional Bands				1							1		1			1			
Anti V16	2				2			3		2			2				2		
Additional Bands								1											
Anti C56	3	1			4	1		2	1	4	1		3	1		1	3	1	
Additional Bands					1					1						1			
Anti C37	2	1			3	1		4	1	3	1		3	1			4	1	
Additional Bands					1			2		1			1				2		
Anti Beverley	1	2	1		2	1	1	2	1	1	2		1	2	1		2	2	1
Additional Bands					1			1									1		

If each zone can be further subdivided, it is possible to obtain greater number of bands representing the antigenic determinants. It is, therefore, an attempt to demonstrate the minimum precipitin bands contributed by each antigen. From the results outlined in table 24, it can be seen that each antigen can be distinguished from the other by the additional band(s) observed on reaction after electrophoresis against different strain specific antisera.

Similar results were obtained when the supernatants were prepared afresh and studied as above.

Experiment 13.

Immunoelectrophoretic study of TC antigens of toxoplasma.

It was of interest to study the electrophoretic mobility of different toxoplasma strain antigens which were free from mouse protein(s) contaminant unlike supernatant antigens. Each strain of toxoplasma TC antigen, after electrophoresis for 75 minutes on microscope slides containing 4 ml agarose/slide was allowed to diffuse against antiRH serum and antiV16 serum separately and the results obtained are given in figure 30. With antiRH serum both RH and V16 antigens gave one distinct precipitin band each, indistinguishable from each other and situated in the same position, possibly $\gamma-\beta$ region. C56, C37 and Beverley gave, rather, an indistinct band apparently similar to RH and V16. With antiV16 serum, all the five antigens reacted similarly, giving 2 precipitin lines each situated approximately in β and $\gamma-\beta$ regions.

Reproducible results were obtained when TC antigens were prepared again and studied.

Experiment 14.

Immunodiffusion study of ten different fractions of each toxoplasma supernatant antigen following electrophoresis.

The antigenic materials obtained on elution from ten different fractions of each strain supernatant following electrophoresis as detailed in materials and methods were allowed to react against rabbit antiRH serum to determine if there were any reaction of non-identity or partial identity among the corresponding fractions of different antigens. Agarose was taken in 4 ml volumes/slide and electrophoresis was carried out for 75 minutes.

From the precipitin bands of each fraction of different antigens, it was not possible to discern any line of partial identity or non-identity (figure 31).

Experiment 15.

Immunodiffusion study of ten different fractions of each strain of toxoplasma TC antigens following electrophoresis.

Using five strain specific TC antigens in place of supernatants, an experiment was conducted on similar lines as above. However, no precipitin lines could be seen in any of the fractions, possibly due to the low concentrations of the TC antigenic materials.

FIGURE 30.

Immunoelectrophoretic study of Tissue Culture antigens of Toxoplasma strain.

1.	Central Trough.		Top Well	Bottom Well
A-C	R antiRH serum	A.	RH TC antigen	V16 TC antigen
		B.	C56 antigen	C37 antigen
		C.	Bev antigen	RH antigen

II.

A-C. R antiV16 serum. A-C. TW and BW - As in I.

A

I. B

C

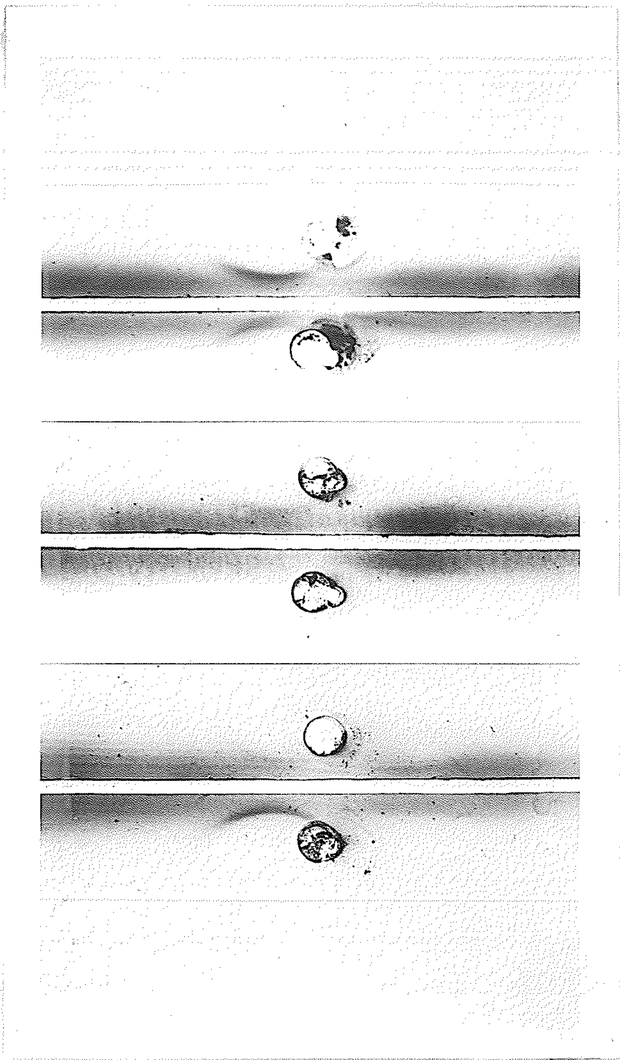


Figure 30.

A

II. B

C

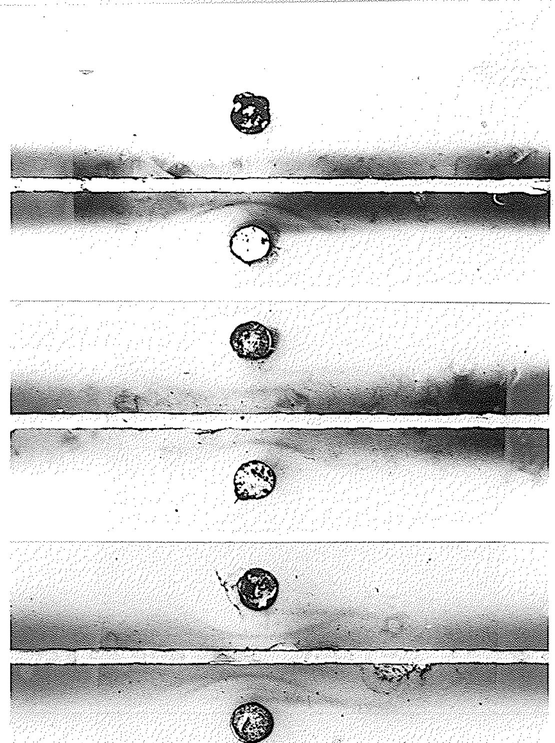
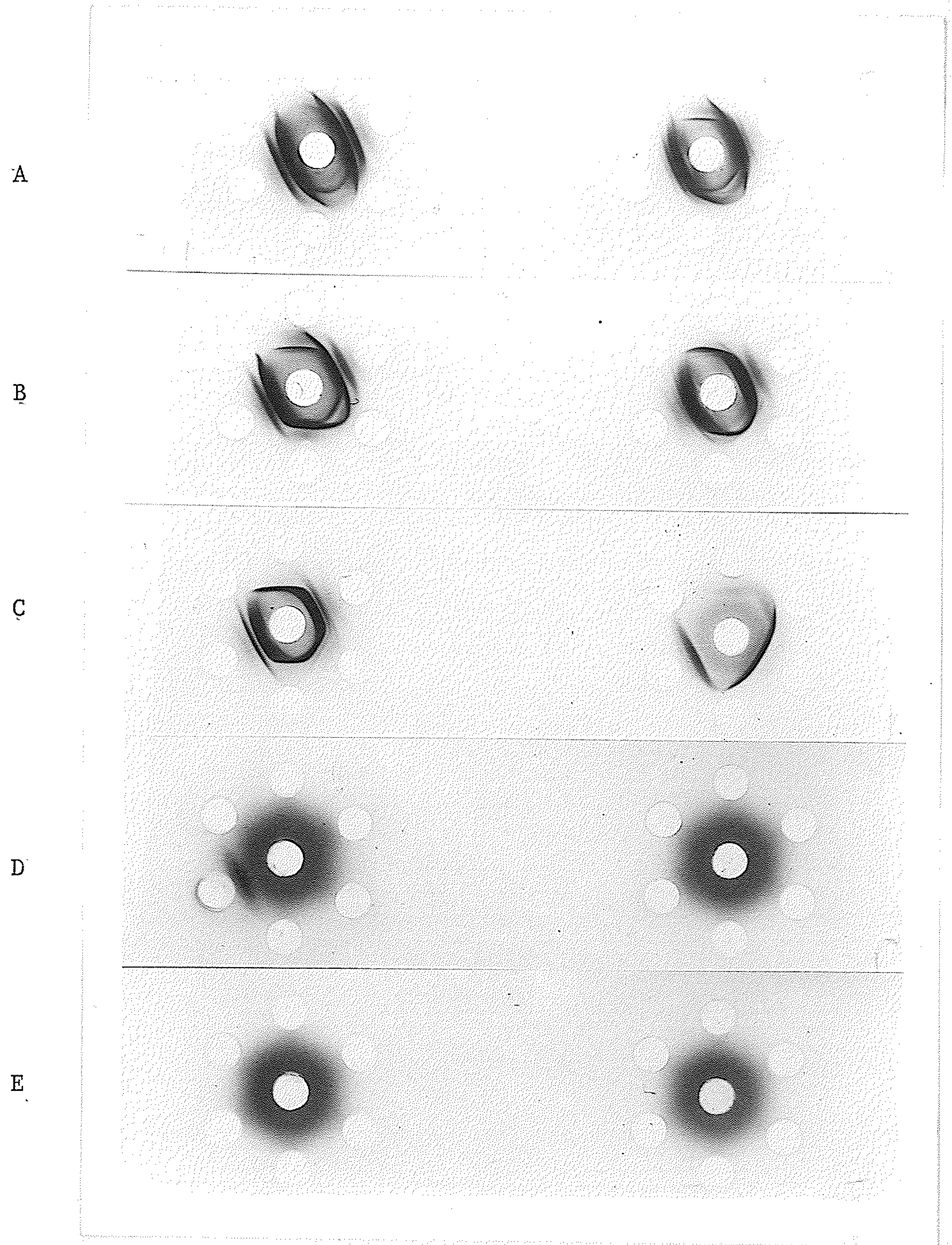


Figure 31

Immunodiffusion study of ten different fractions of each toxoplasma supernatant antigen following electrophoresis.

CW Left & Right	PW Left	PW Right
A-E. R antiRH serum		
	A. Fraction 1 of -1. RH	Fraction 2 of -1. RH.
	2. V16	2. V16
	3. C56	3. C56
	4. C37	4. C37
	5. Bev	5. Bev
	6. Saline	6. Saline
	B. Fraction 3 of antigens as in A.	Fraction 4 of antigens as in A.
	C. Fraction 5 of antigens as in A.	Fraction 6 of antigens as in A.
	D. Fraction 7 of antigens as in A.	Fraction 8 of antigens as in A.
	E. Fraction 9 of antigens as in A.	Fraction 10 of antigens as in A.

Figure 31.



DISCUSSION

NEUTRALIZATION

Neutralization may be defined as loss of infectivity of a living agent through its reaction with specific antibody. The antigenic determinants involved in the reaction would be expected to be located on the surface of the organism, as antigens situated deeply inside may not be accessible to the antiserum.

The methods of neutralization usually employed are:

1) The kinetic neutralization assay, in which a suspension containing an estimated number of virus is mixed with an equal volume of diluted antiserum. Both constituents of the neutralization mixture are equilibrated at 37°C prior to mixing. Neutralization as a function of time is estimated by plaque assay on cell monolayers at timed intervals. When the log relative infectivity is plotted against time, a straight line is invariably obtained, indicating a first order kinetics. Neutralization rate constant or velocity constant (K) of a serum is usually given for the rate when the serum is used undiluted and is calculated from the slope of kinetic curves. When a single serum is tested against a number of virus strains that differ from each other antigenically, K values can be used to determine the relatedness of the strains.

2) Determination of titre of antiserum at 50% end point method: A constant amount of virus and a constant amount of varying dilutions of antiserum are mixed and incubated for a constant time and assayed for infectivity by inoculating, say, ten cell monolayers or ten mice for each dilution of antiserum to find out the dilution of antiserum giving plaques in 50% of cell monolayers or 50% death in mice respectively.

3) Plaque reduction method: plaque assay is carried out to determine the dilution of antiserum giving 80% reduction of number of plaques, that is, the 80% plaque neutralization titre of the antiserum.

4) The metabolic neutralization test based on inhibition of cellular metabolism by a cytopathic effect of a virus as reflected in the pH change in the medium on inoculation of a mixture of virus and antiserum as compared to a mixture of virus and preimmune serum in tubes.

5) The Percentage Law: Antiserum-virus mixtures are assayed without dilution and the relative concentration of antibody is derived from the proportion of neutralized virus. Under conditions of antibody excess, the proportion or percentage of virus neutralized by a given antiserum is a constant, irrespective of the virus dose. It measures both reversible and stable antibody-virion complexes. Although it is not suitable for great accurate measurement, it is considered adequate and is widely used for diagnostic purposes (Andrewes and Elford, 1933a). The "percentage law", according to Andrewes and Elford, is an indication that in antibody excess, the proportionate reduction in the number of plaques is independent of the phage concentration. In Burnet's own words, 'the "percentage law" may break down in extremes of concentration but over the usual range it may be accepted as a working rule which greatly simplifies the design and interpretation of experiments on other quantitative aspects of the reaction' (Burnet, 1933).

It is obvious from the results of experiment 4 that kinetics of neutralization assay is not suitable for toxoplasma since it did not follow first order kinetics with rabbit antitoxoplasma serum plus inactivated complement. With chicken antiRH serum against its homologous strain the

the rate of neutralization for the first two hours seemed to give a straight line but the K value for the antiserum was as low as 0.115. Diwan and coworkers (1963) could not use early sera for the kinetic study of poliovirus for, they yielded very low K values (K = 4) and according to these authors consistent results with heterologous strains could be obtained only when the homologous K values were in the high range. However, in the disk neutralization test, early antisera were found more discriminating in being able to detect antigenic differences between homologous and heterologous strains better than late immune sera. Disk neutralization test was designed by them to measure the degree of neutralization of the virus and they obtained a complete correlation between the results obtained with the disk method and the kinetics test. Neutralization of toxoplasma as determined by the proportion or percentage of surviving plaque-formers that are not neutralized is indicative of the degree of neutralization analogous to the disk neutralization test employed by Diwan and associates for poliovirus. Further, in the present study reproducible results could be obtained in the direct method even as the dose of toxoplasma varied somewhat from one experiment to another and hence the percentage law was applied.

Nikkels (1965) employed passive protection tests in mice by challenging passively immunized mice with graded doses of toxoplasma of different strains and found the method unsuitable for satisfactory differentiation of the strains.

Dilution of antiserum giving 80% reduction of plaques is again not applicable to toxoplasma in view of the rather low levels of neutralization obtained with rabbit antitoxoplasma hyperimmune serum against the five strains of toxoplasma.

Walsh (1950) obtained disappointing results in his attempts to determine the capacity of the human serum containing toxoplasma antibodies to inhibit or modify the skin lesions produced by toxoplasmosis in the rabbit skin. When the serum was diluted even by a small factor, the effect disappeared and this type of test yielded only qualitative information. It was not possible to differentiate between the toxoplasmic antibody present in the sera of normal individuals and that found in the mother or a child who was definitely proved to be infected.

The metabolic inhibition test is not considered a critical test for quantitative differentiation among different strains of toxoplasma and as such the neutralization test by "percentage law" was employed as it appeared feasible, more practical and suitable for both reversible and stable antibody-virion complexes.

In the neutralization experiments, toxoplasma was obtained from the peritoneal exudate of mice and would therefore contain some mouse protein(s). Antisera produced in rabbits from such an antigen after formalin treatment would therefore comprise antibodies to toxoplasma as well as antibodies to mouse proteins. To circumvent this pitfall absorption of rabbit antitoxoplasma serum with lyophilized NMS in order to remove antibodies to mouse protein seems a good and straightforward proposition in theory but in practice such absorption besides removing antibodies to mouse proteins as desired, might result in absorption of neutralizing antibodies of toxoplasma non-specifically as experienced in other systems, for example, Eckert <u>et al.</u>,(1955) working on absorption of antibodies to chicken proteins from rabbit antiavian myeloblastosis serum. Two other alternatives were then considered. One was to use toxoplasma grown

in tissue culture as the source of antigen for neutralization experiments instead of toxoplasma from the mouse exudate. By rule of thumb, it is accepted that this is not a highly practical procedure. Further, it was not encouraging to realize that the yield of toxoplasma is not very great, nor is it easy to decide on an optimal harvest time. Above all, the proportion of nonviable forms in the nutrient medium is very high since there is no synchronicity in the rupture of infected cells and release of toxoplasma in tissue cultures (Jacobs, 1956), and since the parasites released from infected cells to the extracellular fluid are inactivated within 24 hours (Lund et al., 1961).. The second alternative was to purify toxoplasma organisms. Lycke and Lund (1964a) had employed differential centrifugation to purify toxoplasma from the mouse peritoneal exudate and were able to eliminate 90% of mouse cells. A modification of their method was undertaken not only to eliminate mouse cells but also to free the extracellular toxoplasma from the intracellular ones, both of which are contained in the mouse exudate.

During the neutralization time of eight hours there is a possibility that some of the intracellular toxoplasma may become extracellular, may be insufficiently neutralized for a period and thus obscure the results. With these in mind, reciprocal neutralization was carried out after purification of toxoplasma also to find out if there is any significant difference in the proportion of neutralization of different strains as compared with toxoplasma strains used without purification in earlier experiments. Further, using purified toxoplasma in varying concentrations in the standard method of neutralization it was possible to demonstrate that there was no substance in the toxoplasma suspension of mouse exudate

corresponding to Burnet's "specific soluble substance" derived from phage with which Burnet obtained some evidence pointing to reversal of phage-antibody union, the specific soluble substance acting as hapten and blocking the phage-antibody reaction (Burnet, 1933).

Working with CHR-HSV-1 and CHR-HSV-3, two antigenically distinct variants of herpes simplex virus (HSV), Hampar et al., (1968) observed that the specificities of 19S and 7S antibodies may differ and that 19S antibodies may be directed more against type specific herpes antigens, whereas 7S antibodies may be directed moreagainst group specific herpes antigens. That late antisera were somewhat less specific than early antisera was also observed by McBride (1959) in his studies on neutralization of various strains of poliomyelitis. If it were true in respect of toxoplasma also, it should be profitable to use early antitoxoplasma sera containing predominently 19S antibodies to differentiate the strains than to use hyperimmune sera. However, with the early antiRH serum (IFAT 1:32) the percentage of neutralization obtained against its homologous strain being as low as 19.62%, any further consideration to employ early antisera for the differentiation of different strains of toxoplasma was abandoned (vide experiment 11).

The percentage of neutralization obtained with all the strain specific rabbit antisera is rather low. This low level of neutralization may be a true reflection of the concentration of neutralizing antibody, or it may be due to the low plaquing efficiency of toxoplasma, the non-plaquing, non-infective toxoplasma being probably in a position to "block" the neutralizing antibody, or it may be that there are thousands of critical receptor sites for the antibody on the entire surface of each toxoplasma and that for an effective neutralization almost all these

receptor sites may have to be reacted upon by neutralizing antibodies or it may even be due to reactivation of toxoplasma, the union of toxoplasma and neutralizing antibody not necessarily forming a stable complex.

There was no demonstrably significant differences in the proportion of neutralization between purified and non-purified toxoplasma. In this respect it seems to simulate viruses in which the loss of infectivity as measured in the neutralization kinetic test does not require high purification of the virus preparation (Hirst, 1965). It is also an indirect evidence to suggest that the antibody-toxoplasma reaction in neutralization is highly specific.

In the present study antitoxoplasma serum was produced in rabbits against formolinized toxoplasma strains, as with the smallest dose containing about ten live toxoplasma (RH), it was not possible to keep the rabbits alive for more than ten days. The rabbit antitoxoplasma sera obtained by injecting formolinized toxoplasma strains in this study resulted in hyperimmune sera with lower concentrations of antibody. Higher titred rabbit antitoxoplasma sera were produced by Van Nunen and Van Der Veen (1965) using apparently live toxoplasma and simultaneously instituting sulphamethazine therapy for ten days starting from the fifth day of the injection schedule. It would have been probably profitable to follow this procedure in order to obtain higher titred antitoxoplasma sera.

It is clear that reactivation of neutralized toxoplasma occurred on "dilution" and that infective toxoplasma reappeared when the mixture was diluted sufficiently as can be seen from the experiments in which varying concentrations of toxoplasma and constant amounts of antiserum-and-inactivated complement were taken and compared with the direct method without dilution. On dilution of the antigen-antibody-inactivated complement mixture, the proportion of reactivation of toxoplasma was large both when the relative concentration of toxoplasma was high (antigen excess) and when it was low (antibody excess), with the least reactivation or the maximum neutralization being evidenced in the optimum zone simulating the classical complement fixation reaction and having relevance to the lattice theory put forward by Marrack (1938). However, as can be seen, the least reactivation or the maximum neutralization of toxoplasma was obtained in the direct method without dilution which is in an extreme antibody excess zone and the result obtained in this zone is in direct contradiction to the above theory. Since the concentration or dilution of toxoplasma varied two-fold from one test tube to the next one the exact concentration of toxoplasma for maximum neutralization is difficult to estimate but should be located around the maximum neutralization observed on dilution, so much so that, it can be presumed that neutralization obtained at the optimum zone (on dilution) is equal to that obtained in the direct method without dilution. In other words, it is possible that maximum neutralization occurs throughout the antibody-excess zone but that dilution brings about reactivation or dissociation, the proportion of reactivation being the least in the optimum zone. If it were so, then the proportion of reactivation in the direct method which is in extreme antibody-excess zone, should be maximum if it is only possible to

dilute sufficiently and assay for the infective toxoplasma but the impracticability of the experimental study leaves the position unclarified. It can also be argued, on the same grounds, that the same proportion of neutralization probably occurs in the antigen-excess zone as in the optimum zone if there was no dilution by a large factor carried out but again this cannot be experimentally demonstrated. Finally, it is not possible to be positive and emphatic that dissociation of antibody from toxoplasma does not occur in the direct method or in the optimum zone, either while in the test tube or while in contact with the monolayer cells during the period of adsorption. Limitations of experimental practicability do not permit an answer to be obtained to this.

The ratio between toxoplasma and antibody could have been changed by varying the antiserum concentration and keeping the toxoplasma, inactivated complement and the dilution factor constant. This would have given information of dissociation arising out of dilution of antiserum; not of dilution of antigen-antibody-inactivated complement mixture. When antiRH serum was used undiluted and diluted 1-4, the reduction in neutralization of toxoplasma was minimal and was not as great as one would have expected on the basis of neutralization of viruses in general involving a 1-4 change in the concentration of antiserum.

Reactivation was first recorded by Otto and Munter (1922) in respect of neutral phage-antiphage mixtures by dilution. On dilution, similar reactivation was observed by David et al., (1953) with Influenza A (strain WS) when assayed on chick embryo. Working with two group B Arboviruses, Murray Valley encephalitis virus and West Nile virus, Westaway (1965) observed reactivation of neutralized virus on dilution at pH 7.4 to the tune of 0.5 to 14% dependent on the concentration of antibody. Reversi-

bility of inactivation of bacteriophage T4 by its antiserum was reported by Haimovich and Sela (1966). The mechanism of virus neutralization by immune serum is not fully understood. Dissociation seen on dilution serves as a direct evidence that toxoplasma are not killed by antibody during neutralization. Like viruses, neutralized toxoplasma probably can adsorb but cannot penetrate cells.

While "dilution" brought about dissociation of antibody from toxoplasma or reactivation of toxoplasma, variation of the dilution factor per se, in the range of 1-400, to 1-50, does not seem to have influenced the degree of reactivation as high proportion of reactivation can be seen both at 1-400 dilution and at 1-50 dilution. What effect, if any, will a series of sequential small factor dilutions have than a single dilution by a large factor on reactivation of toxoplasma is not known.

One of the important variables can be the host-cell (indicator) system used for assay of survivors of toxoplasma in neutralization. There is evidence that what appears to be completely neutralized, as judged by assay in one host cell system may be highly infective in another cell system (Horsfall, 1957).

According to Floorman and Trader (1947), demonstration of antigenic variation was dependent not only upon the experimental method but also upon the indicator system in which the viral activity was measured. Therefore, it may be said, that neutralization is not the inevitable consequence of certain antibody-virion complexes but results from a triple reaction of antibody-virion-and-host cells. Different residual infectivity can be obtained in different host cells with the same antibody-antigen system.

In the case of toxoplasma, as in a few viruses, guinea pig complement influences to enhance neutralization. The question that naturally crops up is whether the neutralizing antibody to toxoplasma is also a complement-fixing antibody. From what has been observed in this study, the answer is both yes and no. It is a complement fixing antibody in the sense that, neutralization is influenced by complement or components of complement as seen from the results of experiments in which complement, inactivated complement and no complement were used. Neutralizing antibody to toxoplasma is not a complement fixing antibody only because neutralization is manifested, though to a lesser degree in the absence of complement, in the direct method. It is not known exactly how complement and inactivated complement act in bringing about enhanced neutralization of toxoplasma by its antiserum. Apparently complement or inactivated complement acts subsequent to sensitization of toxoplasma by its antibody. This probably involves attachment of the components of complement to specific sites of the sensitizing antibody and/or the toxoplasma. It is also not known why neutralization is greater in the presence of inactivated guinea pig complement than in the presence of complement. It has been explained that in the heat inactivated guinea pig complement the thermolabile inhibitors such as inhibitors of C'4 and C'6 are removed leaving the thermostable C'4 and C'6 free from the inhibitory influence of their respective inhibitors to exercise their effect on the neutralization of toxoplasma by its antibody (Shettigara, 1971). Another plausible explanation is that for enhanced neutralization of toxoplasma, all the components of complement are not required. In the heat inactivated complement the heat-labile C'1, C'2 and C'5 and perhaps the relatively heat-labile

C'8 amd C'9 are destroyed, leaving heat-stable components to act without any competitive interference by other components to bring about enhanced neutralization. This reminds of the unsettled mechanism by which Ig E antibodies fix complement. Recently, Ishizaka et al., (1972) have reported that Ig E myeloma protein aggregated by coupling with bis-di-azotized benzidine was found to activate complement (human and guinea pig) through an alternate pathway, fixing the late components of complement, namely, C'3, C'5, C'6, C'7, C'8 and C'9 but not C'1, C'4 and C'2.

Until more information on the requirement of the exact components of complement, their optimum concentrations and their sequence of fixation is obtained by working with purified components of complement on similar lines as the study of Hampar et al., (1968) on neutralization of HSV by its homologous early and late antisera, any explanation at this stage, would be nothing more than hypothetical.

The use of antigamma globulin serum at a final concentration of 2.5% was found to enhance the neutralization titre of the immune sera and 7S antibody against HSV whereas it had no such enhancing activity with 19S antibody (Hampar et al., 1968). Similar enhancement of neutralization of haptencoupled bacteriophage by antihapten antibody was demonstrated by Makela (1966) and Carter et al., (1968). Makela (1966) employed antigamma globulin serum diluted 1/2 and mixed with an equal volume of 10% antihapten serum. Carter et al., (1968) employed different concentrations of antigamma globulin in nutrient broth to increase the sensitivity of neutralization at least three fold . In the present study, antichicken gamma globulin serum was used in two different dilutions, namely 1-10 and 1-100, final dilutions being respectively 1-30 and

and 1-300. Each of the dilutions of antigamma globulin was added 30 and 60 minutes after incubation of toxoplasma-chicken antitoxoplasma serum mixture. When antigamma globulin was added at 1-10 dilutions there were almost no plaques seen on assay at 30 and 60 minutes later both in the test and control systems after the mixing of toxoplasma-chicken antitoxoplasma serum, whereas, 1-100 dilution of antigamma globulin gave, rather, poor neutralization - namely, less than 25%. While 1-10 dilution of antigamma globulin was apparently toxic to toxoplasma organisms, 1-100 dilution succeeded in producing but poor neutralization of sensitized toxoplasma. The mechanism by which antigamma globulin neutralized sensitized HSV (Hampar et al., 1968) or hapten coupled bacteriophage (Carter et al., 1968) was not known. In the like vein, it is not possible to explain the poor neutralizing ability of antigamma globulin on sensitized toxoplasma by chicken antibody as manifested in the present study. ARGG on the other hand produced an erratic effect on neutralization of toxoplasma by rabbit antibody in the presence of guinea pig complement when the antigamma globulin was used in a final concentration of 1-40 and 1-400 dilutions.

The source of persistent fraction of bacteriophages and viruses in neutralization has been discussed by many authors. Burnet et al., (1937) suggested that it was due to dissociation of the active virus from the inactive virus-antibody complex, implying the obvious existence of heterogeneity among pure phage population. Further, aggregation of phage particles, particularly when their concentration is high and adequate time

is allowed was also considered by them as a possibility. Furth and Landsteiner (1929) and Burnet (1934) have brought evidence for the presence of heterogeneity amongst antibody molecules in the case of bacterial antisera, which according to them, is not a simple reflection of multiplicity of components in the corresponding antigens but arises because each antibody molecule can carry modified superficial groupings corresponding only to a proportion of the number of qualitatively distinct determined groups of the antigen molecule. Recent evidence also points to inhomogeneity of antibody or antibody heterogeneity as the source of resistance to neutralization. Working with poliovirus, Svehag and Mandel (1964) have shown that early antisera leave a much higher fraction of non-neutralizable survivors than late antisera. Lafferty (1963) demonstrated that a degraded antibody is poor in its neutralizing capacity and that it is able to convert virus to a non-neutralizable state. Based on this evidence he proposed that in antisera ill-fitting antibodies do exist and they attach to virus but fail to neutralize the virus; nevertheless, they succeed in blocking the attachment of better-fitting antibodies.

It is well known that evidence of antibody heterogeneity may be obtained by comparing antisera from the same animal at different stages of immunization (Heidelberger and Kendall, 1935). Bearing this in mind, antisera collected at different days from a rabbit were pooled and the experiments were carried out from the pooled antiserum in respect of each strain of toxoplasma.

Dulbecco _et al._, (1956) and Mandel (1961) have identified the persistent fraction with a portion of viral population that is resistant to neutralization.

It has not been possible to appreciate any difference in the size of the plaques among surviving toxoplasma after neutralization as compared with those in the control nor was it possible to appreciate the different strains of toxoplasma by the difference in size of the plaques produced by each strain.

Hsuing and Melnick (1955) reported that the sensitivity of the plaque method in petri dishes and bottle cultures was the same in respect of poliomyelitis, Coxsackie and orphan (Echo) viruses assayed on monkey epithelial cells. Judging from the results this seems to hold true for toxoplasma on Vero monolayers in petri dishes and in T-flasks carried out in this study.

All survival rates are referred to control tubes, and are therefore not affected by the thermal attrition of toxoplasma.

Neutralization test for toxoplasma is not extremely sensitive or delicate unlike neutralization for viruses and bacteriophages but it should be possible to improve upon this when the exact nature of the role of components of complement becomes known, a more sensitive indicator cell system is, perhaps, found and possibly a better buffer system than the one employed in the present study can be availed of. However, for all practical purposes, it is not necessary or desirable to have an extreme sensitivity for, as Horsfall (1957) contended, exquisite sensitivity can be, by itself, a pitfall leading to misinterpretation. Minor antigenic components in one virus may lead to antibodies that can neutralize small amounts of another distantly related virus and suggest similarity. This may be a rare possibility but more likely is that common antigens may be missed in high sensitivity and unrelatedness between viruses assumed when relatedness is, in fact, the case.

In the present study, maximum neutralization of toxoplasma (RH) by rabbit antiRH serum was achieved when the organisms were suspended in HBSS or PBS with a pH of 7.7, The optimum pH of HBSS again was about 7.7 when chicken antiserum was examined for neutralization of RH strain. It is of interest to note that at the end of 4 hours' neutralization using HBSS (1X), the pH of the neutralization mixture in all the tubes, irrespective of their original pH which ranged from 6.8 to 7.95, reached 8.6 to 8.8, indicating that during the process of neutralization, probably CO_2 is given off as also some metabolite(s) of alkaline nature was being produced and that the strength of the phosphates in HBSS was insufficient to keep the pH unchanged or to combat this wide change in pH. When the concentration of phosphates was increased to 4X and 20X, the fluctuation of pH was proportionately narrowed down. However, the increase in osmolarity that followed increased concentrations to 4X and 20X was neither conducive for optimum neutralization as with 4X nor without detrimental effect on the viability of toxoplasma as observed with 20X HBSS.

Strannegard (1967a), in his study on the immunoinactivation of toxoplasma by its antibody and activator factor as judged by morphological alterations of toxoplasma observed either under phase contrast microscopy or by staining with AMB, observed that the optimum immunoinactivation of toxoplasma required a pH 8.5 to 8.7. He arrived at this data by determining the pH at the end of one hour's incubation at 37^0C of the buffered parasite-serum mixture. In the present study at the end of four hours' neutralization in HBSS(1X) the pH in all the different tubes was uniformly 8.6 to 8.8 irrespective of the initial pH of HBSS(1X) which ranged from 6.8 to 7.95. If optimum pH is to be judged at the end of neutralization reaction, then both maximum and minimum as well as intermediate neutralization occurred at about the same pH range in this study.

It would, therefore, be a mistake to be guided by the pH at the end of the reaction for judging the optimum pH requirement. Furthermore, it is important to note that Strannegard (1967a) adjusted the pH of HBSS using sodium bicarbonate solution to obtain different pH range. In other words, there was, in addition to change in pH, a change in ionic strength also in the buffer system employed by him.

In the neutralization experiments using HBSS(IX) and PBS(IX), neutralization to the tune of 82.05 and 68.08% respectively was observed and HBSS(IX) was therefore preferred to PBS(IX). However, in view of the implications of inaccuracy involved in the counting of extracellular, live toxoplasma, it is not possible to be too sure or emphatic on this preference unless the two experiments with HBSS and PBS buffers were run simultaneously using a common toxoplasma suspension pool for both the buffer system experiments. This becomes necessary in the light of the variation in the proportion of dissociation by dilution method, depending on the concentration of toxoplasma in the reaction system.

About the middle of the study period, different groups of mice were freshly inoculated with each of the five strains of toxoplasma that was originally preserved in "freeze medium" containing 10% DMSO (final concentration) at liquid nitrogen temperature and further passages were carried out as before. This was done with a view to overcome the possibility of occurrence of any mutation of toxoplasma strains on continuous passage over a prolonged period of time.

Normally an antiserum would be expected to neutralize its homologous strain greater than heterologous strains. Similarly, an antigen should be neutralized to a greater degree by its homologous antiserum than by heterologous antisera. The latter may not always hold as the titre of one antiserum can vary from the other and a higher titered heterologous antiserum may show greater neutralization than a lower titered homologous

antiserum. However, this can be overcome by converting the absolute percentage of neutralization to normalized percentage which would facilitate comparison of degree of neutralization of a particular strain of toxoplasma by several antisera. On the latter basis, it was found that antiRH, antiV16 and antiBeverley sera neutralized their homologous strains more than other strains but it was not true with antiC56 and antiC37. It is, without doubt, difficult to explain these pitfalls. When judged from the amount of neutralization strain-wise, it can be appreciated that RH, C56, C37, and Beverley were neutralized by their homologous antisera to a greater extent than by the heterologous antisera. V16 stands out as an exception, being neutralized more by antiRH (heterologous) serum than by antiV16 (homologous) serum. When the antisera were analyzed by comparing with each other with respect to their neutralizing capacity of various strains of toxoplasma in all possible combinations, namely ten, (unordered sampling), all combinations showed significance with p between 0.05 and 0.10 and the evidence was not sufficient to conclude that antiC37 and antiBeverley were different ($p > 0.50$). Similarly, when the antigens were compared with each other, RH and V16, RH and C56, RH and C37, RH and Beverley, V16 and C56, V16 and C37, V16 and Beverley, and C56 and Beverley were found to be significantly different. C56 and C37 showed borderline significance with p between 0.05 and 0.10, and C37 and Beverley showed no significant difference ($p > 0.20$). At an arbitrary level of 10 per cent significance, it is only C37 and Beverley strains that cannot be differentiated from each other; likewise, antiC37 and antiBeverley sera.

Ashe and Scherp (1963) were able to make a preliminary grouping of HSV strains following neutralization kinetics by adopting the criterion that combinations giving reciprocal NK values of 90 or more were serologically homologous. Since the percentage neutralization is far less sensitive than neutralization kinetics, normalized neutralization values obtained in respect of each strain-serum combination in the present study do not seem to lend themselves for a similar grouping by this conventional method applied in the kinetics study. Comparison was therefore made on the overall picture of neutralization of each strain of toxoplasma by all the five antisera employing Chi-square test.

When the infected monolayers were washed with diluent after adsorption and compared with unwashed monolayers, no significant difference in the surviving toxoplasma fraction from neutralization mixtures were observed in preliminary studies. Hence, in all experiments the monolayers were not washed for plaque assay in the present study.

While the concentration of antisera about 3X resulted in increased IFAT titres of antiV16 by one-fold and decreased titre by one-fold of other antisera, the proportion of neutralization improved significantly with concentrated antiBeverley serum as compared to non-concentrated antiBeverley serum and the change in the proportion of neutralization were minimal with other concentrated strain specific antisera. Since the decrease of IFAT titre by one-fold when tested after an interval of about a year from the first test does not necessarily indicate any drop in titre, it does suggest that IFAT titre may not have a good correlation with neutralization titre.

In experiments with varying concentration of toxoplasma and constant antibody-inactivated complement, it was observed that there was proportionately greater attrition of toxoplasma during 8 hours' neutralization

in antigen-excess and in antibody-excess zones as compared to that in optimum zone and in the direct method, though not to the same magnitude or extent as the proportion of dissociation observed in these zones. This excess attrition of toxoplasma in the control tubes in antigen-excess as well as in antibody-excess zones is difficult to explain and it is also difficult to assess the extent to which this attrition of toxoplasma might have influenced and obscured the dissociation event.

VIRULENCE

The virulence of a given strain of toxoplasma is usually estimated by means of laboratory experiments with mice. Such studies should be quantitative in order to obtain significant measurements. This can be accomplished by inoculating each group of mice with known numbers of viable toxoplasma within a brief period of time, so that attrition of parasites in the suspending medium does not obscure the outcome. The virulence of a strain can then be estimated by calculating the number of mice dead per number of parasites inoculated for each dilution and the survival period, in terms of days, of those mice that died (WHO, 1969). Quantitative study relating time-to-death to the number of free organisms inoculated, as well as LD 50 (median or fifty per cent lethal dose), might provide a means for making comparative measurements of the virulence of the various strains of *T. gondii* (Eyles *et al*., 1956).

One of the difficulties encountered has been the poor accuracy in determining the count of free, live toxoplasma under the light microscope in the haemocytometer after staining with AMB dye solution (Foley and Remington, 1969; Nikkels, 1965) which is believed to stain only viable toxoplasma. However, as Lelong and Desmonts (1952) had reported, live toxoplasma in hypertonic saline solutions, and live toxoplasma after

freezing do not stain with AMB. In addition, it is possible to observe different shades of staining among different toxoplasma and the parasites can be seen taking positions at different depths in the suspending AMB in the haemocytometer, making it impossible to count the parasites to any satisfactory accuracy. To overcome this inaccuracy it was proposed to account for the number of free toxoplasma inoculated into each mouse in terms of plaques obtainable in Vero cell cultures and estimate LD 50 in terms of plaques than in terms of free zoites, thus maintaining better accuracy. In doing so, it is possible that the difference between strains of toxoplasma is narrowed down than if it were possible to count live toxoplasma more accurately and use their number as such. As a result of this adaptation of expression of LD 50 in terms of PFU, greater accuracy is maintained at the cost of, maybe, sensitivity. Sensitivity is affected to the extent that the variation in plaquing efficiency among different strains of toxoplasma which, it is normal to expect to be extant, is rather "hidden" by the above modification. However, further improvement in the differentiation of virulence among the strains could be always realized if it became possible to develop a method with which to obtain accurate counts of live toxoplasma in the suspensions without much loss of time. The time factor is important since the death of toxoplasma during the period taken for counting, if unduly prolonged, can obscure the final results, the attrition of each strain of toxoplasma in the suspending fluid being likely to vary from one strain to another as a function of time.

Bryan and Beard (1939) estimated the infectivity of purified papilloma virus protein in rabbits by a statistical study of the correlation of amount of virus protein inoculated to the incubation period of induced lesions, and found the relationship to be linear. Their attempts to assay

the infectivity by the 50% end point, (ED50), however, showed that the results were variable and, therefore, not very suitable for papilloma virus protein infectivity assay. Working with influenza virus Horsfall (1939) obtained fairly consistent 50% mortality rate in mice inoculated with serial dilutions of influenza virus suspension intranasally. However, his attempts to utilize the relation between the amount of virus inoculated intranasally and the corresponding survival time as an indication of the potency of the virus preparation was unsatisfactory, as only an approximate indication of the amount of virus inoculated could be obtained by this method for influenza virus, as the variations of the dose of virus were rather high at equal periods of survival. Whereas for the estimation of infectivity, assay by graded response (RT50) was found more suitable than assay by quantal response (ED50) with papilloma virus protein, the converse was found true with influenza virus. A definite linear relationship between the logarithm of dose and the mean reciprocal of the latent period was observed with mouse encephalitis virus by Gard (1940), and with the agent of chicken tumour I by Bryan (1946). Golub (1948) demonstrated the existence of a linear relationship between the average day of death (ADD) at each dilution and the LD50 values in chick embryos using four different strains of psittacosis-lymphogranuloma virus (LGV) group of viruses, making it possible to estimate directly the LD50 value of a virus suspension from the ADD of a group of sufficient number of eggs inoculated with one dilution. A study of the above findings clearly brings out the fact that, depending on the agent to be examined for estimation of its infectivity, either quantal assay or graded response assay or both could be utilized.

In the present study both the LD50 and the RT50 values have been utilized to represent the virulence of a strain and to make a comparative study of the virulence of the five strains of toxoplasma. There is a linear relationship between the concentration of toxoplasma inoculated and the percentage of death of mice within the 100 per cent and 0 per cent range as seen in the five probit curves. It is interesting to find that there also exists a good correlation between LD50 (median lethal dose) and RT50 (median response time) of five different strains of toxoplasma examined in the study, the strain with the smallest LD50 value having the smallest RT50 value, ranging all the way to the strain with the largest LD50 value having also the largest RT50 value. As suggested by Golub (1948), it seems reasonable to consider the relationship between the concentration of toxoplasma strain inoculated and death of mice as being a manifestation of the growth curve of the strain of toxoplasma whereas, the time taken for killing the mice as an indication of the time required for multiplication of a given amount of toxoplasma to reach the critical point at which sufficient of its population to infect and intoxicate and thus kill the mice become possible. Therefore, the product of the two values of LD50 and RT50 in respect of a strain of toxoplasma should as well represent virulence of that particular strain denoting the relative invasive as well as proliferative magnitude of the strain as compared to another strain. This seems justified by the existence of a high coefficient of correlation (Spearman's) ($r_s = 0.903$) between LD50 and RT50 values found in the present study.

The normalized virulence values derived from LD50 ranged from 100 to 16 for the five strains of toxoplasma examined in the present study whereas the normalized virulence derived from RT50 ranged from 100 to 69. This may be mistaken as suggesting that estimation of LD50 of toxoplasma strains gave a better distinction of virulence as compared to RT50. More importantly, however, there was greater consistency in the two values of RT50 in respect of each strain of toxoplasma than the LD50 values as evident from the coefficient of variation for RT50 and LD50 in replicate experiments, namely, 0.035 and 0.429 respectively, making RT50 the method of choice for the estimation of virulence for toxoplasma strains.

Strains have been often considered virulent merely because they caused the death of inoculated mice on primary isolation. This can be misleading as there is way to assess the concentration of parasite in the original inoculum. As soon as the organisms can be quantitated it becomes possible to estimate virulence. This must be done early with a new isolate and any subsequent changes in virulence can be determined subsequently (WHO,1969). The five strains of toxoplasma under study were received either in the form of infected mouse brain or suspended in heparinized blood. None of them being a new isolate, each has presumably undergone several passages in mice or chick embryos or tissue cultures, thus altering the original virulence. In order to standardize them uniformly, each strain was subpassaged in succession in Swiss White mice until the strain reached maximum virulence; in other words, until the strain was "fixed", and the mice inoculated with a given dose of inoculum regularly became morbid at regular intervals of 3 or 4 days, depending on the strain of toxoplasma, further passages in mice having no effect on this interval.

There was wide variation in LD50 values of two experiments in the case of RH, C56 and Beverley strains but not with V16 and C37 strains. It is difficult to explain this variation. Interval between two experiments ranged from 2 to 9 months among the five strains. At least three variables were involved, namely, biological variables, variation from one occasion to another and measurement error. Biological variation should include not only the mouse population but also the Vero cell population. Any one of the three variables or a combination of them could have played a part in affecting the variation. In addition, it may be also argued if, with some strains of toxoplasma, the virulence is subject to fluctuation when being continuously passaged in mice.

Although it is possible to quantitate virulence by estimating LD50 and RT50, virulence is a complex phenomenon, being a composite of the invasiveness and rate of multiplication of the parasite and various host factors, and understandable only in terms of host-parasite interactions. In cell cultures only the "inherent resistance" of the cells and the properties intrinsic to and innately present in the various strains of organisms contribute to the results obtained as no antibody is presumably produced in cell cultures. In the intact animals, on the other hand, all sorts of host defences - physical, humoral, cellular and hormonal - tend to reduce the chances for successful infection. Degree of success in infecting an animal host is believed to correlate with the virulence of a strain in respect of that particular species and breed of the animal and the relative virulence of the strains of toxoplasma examined in this study, therefore, relates to mice but not to any other animal species or to human beings. The various defences encountered in animals being not present in cell cultures, variation in sensitivities between cell cultures and mice to toxoplasma can be naturally expected. In this con-

nection it is pertinent to note that Abbas (1967) found that mice inoculated intraperitoneally were at least 16 times as sensitive as chick embryos and at least 316 times as sensitive as HEp 2 and He La cells for isolation of toxoplasma from acutely infected tissues, and were 10 times superior to chick embryos in sensitivity for isolation from chronically infected tissues, cell cultures being of little value in the latter case. The relative higher sensitivity of mice as compared to cell cultures might explain the smaller number of PFU obtained as LD50 in respect of different strains of toxoplasma. A more sensitive cell than Vero cells would be an advantage in magnifying the difference in LD50 of different strains of toxoplasma in terms of plaques.

While it has been accepted that some strains of toxoplasma in vivo are more lethal than others (Kaufman et al., 1958; Jacobs, 1968) and while there is some evidence that virulent strains may be slightly larger in size than avirulent strains (Harboe et al., 1955), there has been no quantitative antigenic, enzymatic or other factor explaining this difference among strains reported. Increase in virulence of strains with continuous animal passage (Jacobs et al., 1954; Sabin and Olitsky, 1941; De Rodaniche, 1954a and 1954b), relatively stable virulence in chick embryo (Jacobs et al., 1954) and in tissue cultures (WHO, 1969), sudden increase in virulence on changing of animal species for passage (Lainson, 1955) have been described, but the nature of this change or adaptation is still obscure.

Peritoneal exudate of mice infected with toxoplasma contains, in addition to free organisms, some parasitized exudate cells, though in small numbers (Eyles et al., 1956). These parasitized exudate cells should

not form plaques in cell cultures by virtue of the fact that toxoplasma within exudate cells are not free and thus have no access to cell cultures to infect the cell monolayer. However, it is difficult to assess their influence on the relationship derived in the latent period of different strains of toxoplasma but it is believed to be minor on account of the small number of these parasitized exudate cells in relation to the number of free toxoplasma (Eyles et al., 1956). No attempt was made at purification of toxoplasma for this aspect of the study in view of the time required for any purification procedure, irrespective of the merits and demerits of these procedures, and the influence of time factor on the attrition of different strains being likely to be at different levels, the final outcome is more than likely to be obscured by attempting to purify the organisms.

In LD50 experiments an attempt should be made to determine whether the surviving mice had sero-conversions (WHO, 1969). In one of the LD50 experiments with RH strain, about 50% of the surviving mice were bled and their sera found negative when tested by IFAT. No further attempts to examine the sera for toxoplasma antibodies were made and it is, therefore, not possible to rule out the possibility of finding titres in survivors in other strains of toxoplasma which are less virulent than the RH strain. However, mouse being the only animal which possesses neither the non-specific antibody nor the activator system, has been found an ideal animal for isolation studies (Feldman, 1968) and for the same reason, probably, in mice the evidence of immunity is an increase in survival time unlike other animals in which a high degree of immunity, generally, develops following survival of toxoplasmosis (Jacobs and Melton, 1954). In the

present study, the mice were observed for 20 days after inoculation and death in mice always occurred within the first 16 days with all the five strains.

Mice of both sexes were used at random for the study as sex of mouse does not seem to play any part in its sensitivity to experimental toxoplasmosis (Nikkels, 1965). According to the observations of Nikkels (1965), both age and weight do not seem to influence the sensitivity of mice to experimental infection with toxoplasma to any degree. However, in the study Swiss White mice weighing 20 to 28 gms in the age group of 6 to 12 weeks were selected since extreme variations in weight and age are not desirable for fear of any fluctuation in sensitivity to toxoplasma. Biological heterogeneity to the tune of about 5% was reported by Nikkels (1965) as evidenced by the number of mice that resisted large doses of toxoplasma inoculations.

Random samples of sera from Swiss White mice employed for virulence test in the study have been examined by IFAT and were found to have no titres for toxoplasma antibodies.

The five strains of T. gondii under study afforded no difficulties whatever in having their virulence "fixed". However, if the study were to involve toxoplasma strain of much lower virulence, it might become necessary to institute corticosteroids or immunosuppressive drugs prior to inoculation of toxoplasma into mice.

IMMUNODIFFUSION AND IMMUNOELECTROPHORESIS

Results of preliminary experiments on double diffusion in agar gel using chicken antiRH serum and rabbit antiRH serum against three types of RH and V16 antigens, namely, sediment, supernatant and lysed antigen, revealed that chicken antiserum was not suitable for immunodiffusion studies of toxoplasma antibodies as no precipitin lines were seen against all the three types of toxoplasma antigens. But the latter was not tested for precipitin bands at higher salt concentration. Rabbit antitoxoplasma serum, on the other hand, gave one thick line of identity between RH and V16 antigens, comprising 3 or 4 bands of precipitation. Sediment and supernatant gave the maximum number of bands of precipitation as compared to DW lysed antigen.

These findings are in agreement with the observations of O'Connor (1957a and 1957b) and Strannegard (1962). However, it must be stated that the DW lysed antigens contained about 200μg protein/ml whereas the supernatant antigen preparations contained well over 5 mg/ml on estimation by Lowry's technique (1951), so much so that the poor reacting ability of DW lysed antigens might be due to the poor concentration of antigenic material than the type of preparation per se.

All the investigators, namely O'Connor (1957a & 1957b), Korting (1958), Strannegard (1962), Spano & Dardanoni (1969) and Hubner and Uhlikova (1969) have worked with toxoplasma antigen obtained from mouse peritoneal exudate and antisera produced against this antigen in rabbits. The number of precipitin lines obtained by them by using such a system would naturally represent not only toxoplasma antigen-antibody complexes but also mouse protein(s) - antimouse protein antibody(s) complexes; the

latter, somehow, or other, had escaped attention and had not been considered by most of them. Strannegard (1962) observed two lines of identity between human and rabbit antisera and he obtained more precipitin lines with rabbit antisera than with human antisera. While antibodies in human antisera from the general population may not contain antibodies to mouse proteins, rabbit antisera should be suspected to contain antibodies to mouse protein as they were produced against toxoplasma contaminated with the mouse protein(s). It stands to reason, therefore, that the additional lines obtained by Strannegard (1962) with rabbit antisera may not actually depict the true immunogenic picture of toxoplasma antigen-antibody system. It is surprising that Strannegard (1962) observed no lines of precipitin when control antigens were used in immunodiffusion against rabbit antitoxoplasma sera. The control antigens employed by him comprised peritoneal exudate from mice which had been inoculated with Ehrlich's ascites tumour cells and treated similarly as the test antigens, namely sediment and supernatant antigens. In the present study, presence of mouse protein in the antigen preparations and of antibodies to mouse proteins in the rabbit antitoxoplasma serum have been clearly demonstrated both in agar-gel diffusion and in immunoelectrophoresis. It is not clear if the negative results obtained by Strannegard with control antigens was due to the fact that the concentration of mouse protein antigens was too low possibly because, as Strannegard himself pointed out, the control antigen could not be concentrated to the same degree as the test antigens, the control exudate being viscous and haemorrhagic in nature.

Korting (1958) reported that toxoplasma antibodies migrated with gamma globulin fraction of rabbit serum producing only one line of precipi-

tation with a sharp edge on one side on electrophoretic separation of antiserum whereas electrophoretic separation of toxoplasma antigen showed at least four separate precipitin lines with homologous antibody, the precipitin lines being situated in albumin, α-1, α-2, and β-2 regions and possibly a fifth in β-2 to γ region. Similar results were obtained in the present study. However, these findings are likely to be obscured by the presence of mouse protein antigen and its antibody. An attempt was, therefore, made to absorb the rabbit antitoxoplasma serum with NMS dry powder (lyophilized) and with NMS-immunosorbent to absorb the antibodies to mouse protein(s) but this procedure in respect of the present system, appeared to be cumbersome, consuming large amounts of antisera in the process and not highly dependable, non-specific absorption being apparently involved in this. Similarly, in another experiment, when rabbit antiRH serum was absorbed with graded amounts of different strains of toxoplasma pellet and rabbit antiV16 serum with graded amounts of RH strain pellet, total absorption of all the antibody components was encountered. Since the different strains of toxoplasma pellet would be naturally contaminated with mouse exudate protein(s), the latter would have accounted for the result obtained.

Eckert <u>et al</u>., (1955) have reported similar non-specific absorption phenomenon when they attempted in several experiments to absorb antibodies to chicken tissue from rabbit antiserum produced against avian myeloblastosis virus from chicken tissue. After absorption with chicken tissue, they encountered a reduction of 67% in complement fixing (CF) antibody titre and a 85% reduction in neutralizing antibody activity. However, in a later report, Eckert <u>et al</u>., (1964) employed Tweenether

treated avian myeloblastosis virus for antisera preparation in rabbits, and the antisera after absorption with chicken tissue, lost all significant reaction with chicken tissue but only a two-fold reduction in serum titre to the virus when tested by CFT. This suggests that any slight change or flaw in the method or technique of absorption may result in complete non-specific absorption.

Ourth (1971a) was able to obtain monospecific antibody to a single antigen-antibody complex of _T. gondii_ by adding gradually increasing amounts of dried NMS to an aliquot of rabbit antitoxoplasma serum, until no lines were observed on the NMS antigen slide after IEP. This seemed to indicate to them that _T. gondii_ produced one parasite band. While the monospecific antibody, without any doubt, belonged to a single antigen-antibody complex of _T. gondii_, it is difficult to conclude that _T. gondii_ produced only one precipitin band if we only do not lose sight of the fact that there are several pitfalls inherent in the absorption procedure. Attempts to absorb an antiserum with an antigen containing multiple antigenic determinants in differing concentrations are invariably attendant with difficulties; complete absorption of antibodies to individual constituents will obviously be achieved with differing quantities of the antiserum and the absorbing material (Osserman, 1960). In addition, non-specific absorption is a pitfall at times inherent in the absorption procedures, and, indeed, difficult of circumvention. In this context, it is of interest to point out that while it was not possible to demonstrate any additional antigenic determinants in the various strains of toxoplasma supernatants compared to NMS in immunodiffusion against antiRH serum, additional arcs were seen when the different strain supernatants

of toxoplasma were run side by side NME and allowed to react with antiRH and antiC56 serums separately, suggesting that there is a difference between NMS and NME in their antigenic constituents and that NME would be the proper material for absorption of antibodies to mouse protein(s) from rabbit antitoxoplasma sera as these antisera were prepared against toxoplasma strains obtained in the mouse peritoneal exudate and not from mouse serum. Further study was then carried out employing TC toxoplasma strain antigens which were found to be free from any mouse protein contamination as evidenced by the absence of any precipitin line when the tissue culture toxoplasma antigens were allowed to react with antimouse serum in agar gel diffusion. As a reference TC control antigen obtained after inoculation of TC with NME in place of purified toxoplasma from mouse peritoneal exudate and treated similarly as the test antigens was employed. It was thus demonstrated that rabbit antitoxoplasma sera contained precipitating antibodies directed to toxoplasma as evidenced by the precipitin bands given by the five strain specific TC antigens while reacting with antiRH and antiV16 sera in immunodiffusion and IEP. The presence of specific antibodies directed to toxoplasma has been conclusively demonstrated in the indirect microRIA using TC RH antigen. Further these results are in agreement with the findings in IEP in which additional bands were encountered with each strain antigen-serum combination as compared to the bands obtained with NME-serum combination, the latter being suitably employed as a reference.

The antibody response to infection with T. gondii is usually studied by means of DT-, CFT-, and IFAT- titres of the sera of hosts under scrutiny.

Disadvantages of CFT, however, are that the animal sera are quite often anticomplementary and cross-reactions have been also encountered in CFT. These do not occur in DT but this test requires live toxoplasma with its attendant hazards, besides requiring accessory factor. These factors have contributed to the fact that DT has remained confined to highly specialized laboratories. IFAT, while being considered as highly specific and almost as sensitive as the DT, is still in its infancy and needs fair amount of standardization; in other words, it is a valuable test in the hands of trained personnel. Agar-gel diffusion technique, even as it was introduced for investigations concerning the antigenic structure of toxoplasma and the immune response to infection with this protozoan by O'Connor in 1957, seems to be well suited by virtue of its resolving power, simplicity and economy to be employed as a diagnostic tool. Hubner *et al.*, (1970) reported to have obtained 90% agreement between positive MPA and isolation of *T. gondii* in their investigations and they found it much superior to DT correlation with isolation which was as low as 39%. That the DT-, CF-, and precipitating- antibodies against toxoplasma are each distinct has been evident from the poor correlation obtained between them by Strannegard (1962). Diagnostic investigations of toxoplasma infection needs besides demonstration of antibody titres, isolation of the parasite to be more meaningful and reliable. However, there are some flaws in both the investigative procedures. There have been reports of isolations of toxoplasma in the absence of antibody titre, and in the presence of low antibody titre. Similarly, failure to isolate toxoplasma from animals with high antibody titres in DT, CFT, or IFAT has

been common experience among workers. While the majority of isolations are from serologically positive animals, a few isolations are reported from serologically negative animals (Eyles, 1952; Jacobs et al., 1952). Finally, there are strains which do not or cannot multiply in the mouse or after a preliminary phase of proliferation, are destroyed by phagocytes or antibodies (Werner, 1967).

Hubner and Uhlikova (1970) tested the sera of mice which were experimentally inoculated with toxoplasma killed by repeated freezing and thawing and obtained negative results in MPA and positive titres in the DT. They, therefore, concluded that MPA was an indicator of non-sterile immunity detecting presence of live toxoplasma in the acute and chronic phase of toxoplasmic infection. This is in contrast with the findings of Strannegard (1962) who obtained faint precipitin lines with repeated subcutaneous depot doses of killed T. gondii grown on the chorioallantoic membrane (CAM) into rabbits. It is also at variance with the findings in the present study wherein precipitin lines were obtained using antisera from rabbits inoculated with formolinized toxoplasma in immunodiffusion and IEP, though it is important to distinguish the precipitin arcs due to mouse protein contaminant and its antibody from the precipitin lines contributed by toxoplasma and its antibodies. It is, however, possible that mouse antisera may differ from rabbit antisera in its response to killed toxoplasma antigens. Even in rabbits, Strannegard (1962) reported obtaining 1 or 2 faint lines of precipitin only after 3 months have lapsed from the time of first injection of killed toxoplasma from CAM, as against an average of 3 or 4 precipitin lines and, in some cases 6 to 7 lines of precipitin obtained earlier than three months with antisera from rabbits

immunized with live toxoplasma from mouse exudate. It is not clear as to how many of the precipitin bands observed in the latter case were due to the mouse protein contaminant and its corresponding antibodies, thus rendering the increased number of precipitin bands observed of dubious value. On the other hand, the rabbit antisera against killed toxoplasma from CAM should not contain any antibody to mouse protein(s) and therefore may truly depict precipitin lines due to toxoplasma antigen and its antibodies.

Study on the dynamics of toxoplasma antibodies in rabbits using living toxoplasma for immunization as carried out by Strannegard (1962) revealed that while maximum concentration of DT- and CF- antibodies was reached in four weeks, the precipitating antibodies appeared later (maximum concentration in about 12 weeks), and disappeared earlier than the DT- and CF- antibodies. Strannegard also observed that some sera did not form any lines of precipitation in spite of a high DT titre of 1/8,000 or a CFT titre of 1/128. On the contrary, some sera negative in CFT were found positive in gel-diffusion tests indicating that precipitating antibodies to toxoplasma were different from DT and CF antibodies, and that negative precipitation test may not be of much value. However, on electrophoresis and elution of rabbit antitoxoplasma sera, he found that the DT- as well as CF- antibodies were located in the same region as the precipitating antibodies, namely in β-2 globulin region.

It is surprising that the supernatant should be such a good antigen even as the sediment contained all the intact toxoplasma unless the supernatant contained soluble antigens. Strannegard (1962) surmised that it

could be due to disruption of toxoplasma either in the mouse abdominal cavity or during centrifugation, or that the antigenic factors may be metabolic products or even soluble toxins from the parasites. A further plausible explanation would be that if an antigen molecule is cleaved in the process of its preparation, it is possible to obtain several precipitin lines (Grabar, 1957). In the case of supernatant antigens, the preparation involved lyophilization after repeated freezing and thawing unlike the preparation of sediment antigens. It is, without doubt, difficult to hazard the guess as to what effect this additional procedure would have had on the antigenic molecules of either toxoplasma or mouse protein(s), if any.

With regard to the preparation of antitoxoplasma sera in rabbits, earlier attempts to obtain satisfactorily titred sera using formolinized toxoplasma grown in CEF tissue cultures were to no avail as the titre of the antisera did not exceed 1-32 in IFAT. When living toxoplasma from mouse exudate was injected subcutaneously into rabbits, even as few as about ten toxoplasma(RH) killed the rabbits within 10 days. On the other hand living toxoplasma from the mouse exudate was successfully employed in chickens and rats for antisera preparation (Shettigara, 1971). For the preparation of antisera in rabbits it, therefore, became necessary to employ formolinized toxoplasma from mouse exudate as described in materials and methods. Strannegard (1962) had successfully employed living toxoplasma from mouse exudate to obtain high titred antisera in rabbits, though a good number of deaths among rabbits as early as two weeks following injections was reported. One striking point of difference, how-

ever, was that he was not able to get completely DT-negative rabbits, that is, rabbits free from natural infection, which fact could be a serious drawback in an immunological investigation involved in differentiating the various strains of toxoplasma such as undertaken in the present study.

CONCLUSION

Reciprocal in vitro neutralization and plaque assay in Vero nonolayers applying the "percentage law" as carried out in the present study could be a method of choice for classifying the various strains of toxoplasma. The method holds great promise with further improvement. Major improvement should follow investigation into the requirement of the exact components of complement and their optimum concentrations. Obtaining higher titred antisera than the ones used in this study should also enhance the usefulness of the method. The method has practical application in determining strain specificity whether in etiological studies of certain specific, clinical entity or in epidemiological investigations involving the extent of spread, source of infection and in relating transmission from index cases to others in an outbreak of toxoplasmosis.

The study of the virulence of toxoplasma strains in mice through determination of LD50 in terms of plaque numbers and RT50 in terms of days leading to derivation of normalized virulence of these two values as well as a normalized virulence value obtained from the product of reciprocals of LD50 and RT50 gave sufficient indication of a method good enough to elicit evidence of strain specificity among toxoplasma strains. This metod appears to have merit in being able to characterize the strains into separate groups. However, fluctuation in virulence of some strains of toxoplasma on continuous passage in mice is an important pitfall. This drawback may possibly be overcome by using toxoplasma strains maintained in the yolk sac of chick embryo, in which toxoplasma is believed to maintain a sustained, low pathogenicity. However, the likelihood of some strains becoming nonpathogenic to mice in this process cannot be ignored. Estimation of virulence of toxoplasma strains, it is

hoped, would help solve the differences in opinion on the pathogenesis and dissemination of congenital and acquired toxoplasmosis in humans in different geographic areas.

Immunodiffusion as a method was found not suitable for differentiation of the precipitin bands of the five strains of toxoplasma antigens in their reactions with strain specific antisera. On the other hand, IEP provided a tool for distinguishing the five strains by the immunoelectrophophoretic mobility of the bands. However, it is of limited utility for classifying the strains of toxoplasma into distinct groups.

Within the limitations of the errors inherent in the experimental methods employed to examine strain specificity among the five strains of toxoplasma, it has been possible to conclude that C37 and Beverley strains are distinctly different from RH, V16, and C56 strains as suggested in neutralization tests. Similarly, V16 and C56 as well as C37 and Beverley appeared to be consistently indistinguishable from each other in their virulence unlike all the other eight combinations of two antigens as depicted by their RT50 values in two experiments carried out in respect of each strain. Evidence for strain specificity, each strain as having a distinct entity was clearly demonstrated in immunoelectrophoretic studies.

SUMMARY

SUMMARY

In the past, several workers have unsuccessfully attempted to classify the various strains of toxoplasma isolated from both mammals and birds. Thus, to date, all strains of toxoplasma are considered to belong to one species of T. gondii.

Previous attempts at classification of T. gondii strains include:

(1) in vitro cross neutralization test using human and hare strains as antigen,

(2) dye test employing rat antisera against various strains of toxoplasma,

(3) passive protection tests in mice,

(4) in vitro neutralization of toxoplasma followed by inoculation of groups of mice for death rate and survival time, and

(5) poly-acrilamide-gel electrophoresis of toxoplasma antigen from different strains.

In the present study three different approaches were made. First, reciprocal in vitro neutralization and plaque assay in Vero monolayers applying the "percentage law" were carried out. Second, the relative virulence of the strains was studied by determining LD50 values in terms of plaque counts and RT50 values in terms of days in respect of each strain as well as by estimation of the product of the reciprocals of these two values. Finally, the strain-specific precipitating antiboides were

examined in immunodiffusion and immunoelectrophoresis.

From the percentage of neutralization of toxoplasma(RH) by its homologous antiserum produced in rabbits, as determined by plaque assay on Vero monolayers it was shown that rabbit antiRH serum does bring about significant neutralization of RH strain on direct assay without dilution but not on dilution and assay. Addition of guinea pig complement resulted in enhanced neutralization of toxoplasma. Further improvement in neutralization was seen in the presence of heat inactivated guinea pig complement. HBSS (1X) with pH 7.7 gave maximum neutralization as compared to HBSS (1X) with pH 6.8, 7.3 and 7.95. Similarly, Dulbecco's PBS with pH 7.65 showed better neutralization than when the pH was 7.8, 7.0 and 6.5. Optimum time for neutralization was determined to be 8 hours and the dose of inactivated guinea pig complement for maximum neutralization was estimated at 8 C'H50 units. The proportion of neutralization obtained with early antiRH serum (IFAT 1:32) was very low as compared to late antiRH (IFAT 1:1024) serum and hence late antisera were used in the neutralization tests. Rabbit antimouse serum did not appear to affect the viability or plaque-forming ability of toxoplasma obtained from mouse peritoneal exudate.

Dilution of antiRH serum 1-4 resulted in lower percentage of neutralization of all the five different strains but the difference was not significant. Dilution method unlike direct method did not give consistent proportion of neutralization, significant differences in neutralization being encountered in some strain-serum combinations. This observation led to neutralization experiments with varying concentration of toxoplasma and constant antiserum-and-inactivated complement by dilution method

which showed that there was dissociation of toxoplasma from its antibody on dilution both in the Ag-excess and Ab-excess zones. Dissociation was minimum at the optimum zone, in which the percentage of neutralization obtained was comparable to that in the direct method. When toxoplasma was purified by differential centrifugation and then employed in neutralization tests, the evidence indicated that there was no soluble factor(s) in the toxoplasma suspension (of non-purified toxoplasma suspension) influencing dissociation of toxoplasma on dilution.

Reciprocal neutralization of five strains of toxoplasma with five strain specific antisera in the presence of inactivated complement as carried out by the direct method revealed on analysis with Chi-square test that at an arbitrary level of 10% significance the five strains could be classified into four groups, C37 and Beverley forming one group and RH, V16 and C56 strains forming three separate groups.

Relative virulence of the five strains of toxoplasma was examined by their LD50, RT50 and the product of these two values in Swiss White mice. Using probit transformation LD50 was obtained in terms of plaque numbers as determined on plaque assay instead of toxoplasma numbers since the latter count under the microscope was fraught with inaccuracy, and hence not dependable. RT50 was obtained in terms of days using Rankit transformation. From the reciprocals of these two values, normalized virulence for each strain was derived separately. Normalized virulence was also estimated from the product of reciprocals of LD50 and RT50. From these three normalized virulence values thus obtained in respect of each strain in two experiments per strain, it was found possible to differentiate all the strains from each other except V16 from C56, and C37 from Beverley.

Immunodiffusion study employing supernatant antigenic preparations from mouse peritoneal exudate and strain specific antisera produced in rabbits, it was not possible to distinguish the strains. All the strains seemed to share a common, thick, diffuse precipitin band, comprising 3 or 4 lines but a similar picture was obtained with NMS in its reaction with the antisera. However, additional precipitin lines became evident when NME was used as a reference. Absorption of antitoxoplasma sera with lyophilized NMS at increasing ratio resulted in complete disappearance of all precipitin bands. Similar results were observed when attempts were made to absorb antiRH serum separately with different strains and antiV16 serum with RH strain. Absorption of antitoxoplasma serum with NMS-immunoabsorbent did not reveal any precipitin line attributable to toxoplasma only.

Antigens prepared from tissue culture in respect of each strain reacted with antiRH as well as antiV16 serum on immunodiffusion to give a diffuse common precipitin band. This provided evidence for the presence of precipitating antibodies in antitoxoplasma sera directed to toxoplasma since the tissue culture antigens were shown to be free from mouse protein(s) contamination. Further, the presence of specific antibodies directed to toxoplasma in antiRH serum was demonstrated in indirect micro-RIA. In the same study evidence for non-specific absorption of toxoplasma antibodies from antiRH serum on absorption with lyophilized NMS was forthcoming.

Immunoelectrophoretic study of the supernatant antigens seemed to provide a method of distinguishing the five strains by the electrophoretic mobility of the bands that were found in addition to those in NME which

was used as control, in their reaction with strain specific antisera following electrophoresis.

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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 200μg/ml., Amphotericin B.2.5μg/ml. (PSF), (100X)	10.1 ml.
Store in freezer	

@ Dr. A. M. Wallbank, personal communication

* Grand Island Biological Company

\+ Difco Laboratories

APPENDIX B.

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 C.

Earle's Balanced Salt Solution (EBSS 10X)

Solution A (10X)

NaCl..............................	34.0 gm
KCl	2.0 gm
$NaH_2PO_4 \cdot H_2O$......................	0.7 gm
Glucose...........................	5.0 gm
Distilled Water qs.	500.0 ml

Solution B (10X)

$CaCl_2$..............................	1.0 gm
$MgCl_2$ $6H_2O$	0.85 gm
Distilled Water qs	500.0 ml

Store A and B in refrigerator; add 500 ml each of A and B to get 5X. (Add B slowly with constant stirring). Autoclave at 121°C for 30 minutes.

APPENDIX D.

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 ½ 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	

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* Dr. A. M. Wallbank, personal communication

APPENDIX E.

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.
$Na_2B_4O_7$ (1.91% aqueous solution)	0.27 ml.

The Na_2CO_3 and $Na_2B_4O_7$ 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 F.

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

Sodium chloride	80.0 gm.
Potassium chloride	2.0 gm.
Na_2HPO_4 (anhydrous)	11.5 gm.
KH_2PO_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 Cl_2	5.0 gm.
Water, q.s. to	500.0 ml.

Solution-C (100X)

Mg Cl_2 6 H_2O	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 μm 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.
$MgSO_4 7H_2O$	2.0 gm.
Na_2HPO_4	0.5 gm.
Glucose	10.0 gm.
KH_2PO_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)

$CaCl_2$	1.4 gm.

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

Solution-C, 2.8%

$NaHCO_3$	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.

OUCHTERLONEY TECHNIQUE OF IMMUNODIFFUSION.

1. Preparation of agarose gel

Reagents: Tris (hydroxymethyl) amino methane (Mann Laboratories, Ultra-pure)
Sodium chloride, reagent grade
Hydrochloric acide (reagent grade) 0.1N
Agarose (Mann Laboratories, Special Grade)

Solution A: Dissolve 9.6912 gm Tris in 1 litre distilled water. Titrate to pH 7.6 with 0.1N HCl (approximately 540 ml). Make volume to 2 litres.
Solution B: Dissolve 11.70 gm Na Cl in 1 litre distilled water (0.2M)
Solution C: (0.04M Tris-buffered saline, pH 7.6):
Mix 1 volume of Solution A with 1 volume of Solution B; check to confirm pH= 7.6

0.9% Agarose: Dissolve 2.70 gm agarose in 300 ml of solution C by heating in boiling water bath. When dissolved, add 1 ml 0.1M sodium azide per 100 ml agarose solution (as preservative). pH measured at temperature of 56°C is 7.1-7.2.

Distribute in 20 ml aliquots in screw-capped containers and store in refrigerator.

APPENDIX K.

Staining of Agarose Slides by Amido Black.

Reagents.

1) 10% glacial acetic acid in 90% ethanol.

2) 10% glacial acetic acid in double distilled water.

3) Amido Black stain- Amido Black 10B 0.5 gm
Mercuric chloride 5.0 gm
Glacial acetic acid 5.0 ml
Double Distilled water qs 100 ml.

Method.

1) Wash slide in borate-saline(large volume, 300 ml) for 24-48 hours with several changes of wash fluid, to wash out excess antigen and antibody.

2) Dry the agarose slide by placing wetted filter paper strips over the wetted agarose and letting air-dry overnight at room temperature.

3) Fix the alide by immersion in 10% glacial acetic acid in 90% ethanol for 10 minutes.

4) Stain in Amido Black stain for 10-15 minutes.

5) Decolourize to remove excess stain from agarose by agitation in 10% glacial acetic acid in double distilled water for 10 minutes. Repeat #5 once or twice.

6) Wash in distilled water and air dry at room temperature.

APPENDIX L.

<u>Borate-saline buffer</u>.

0.1M boric acid .... 100.0 ml

Adjust pH to 8.0 with 1N Na OH.

Add physiological saline (0.85% Na Cl).. 1900.0 ml

APPENDIX M.

Barbital-acetate Buffer pH 8.6

Sodium barbital	10.8 gm.
Sodium acetate, $3H_2O$	8.6 gm.
0.1 N HCl	116.4 ml.
Distilled water, qs to	2000.0 ml.
Adjust pH to 8.6	

APPENDIX N

T. gondii strain RH - Details of plaque numbers obtained and percentage of mice which died in respect of each dilution of toxoplasma suspension.

Experiment Number.	Serial Dilution Number.	Dilution Factor.	Plate #1.	Plate #2.	Plate #3.	Plate #4.	Plate #5.	Average per plate.	Per cent Mice Dead.
1.	A.	Undil.	64	80	61	70	62	67.4	N.D.
	B.	1-2.	36	33	31	32	29	32.2	N.D.
	C.	1-4.	20	17	17	19	20	18.6	N.D.
	D.	1-8.	7	7	8	6	8	7.2	N.D.
	E.	1-16.	3	4	3	4	1	3.0	N.D.
	1.	1-32.	1	1	3	3	2	2.0	100.
	2.	1-64.	2	1	1	2	1	1.4	70.
	3.	1-128.	2	1	1	1	1	1.2	70.
	4.	1-256.	0	0	0	0	0	0.0	30.
	5.	1-512.	0	0	0	0	0	0.0	30.
	6.	1-1024.	0	0	0	0	0	0.0	0.
	7.	1-2048.	0	0	0	0	0	0.0	0.
@.	A	Undil.	23	26	13	x	x	20.67	N.D.
	1.	1-4.	8	2	9	5	2	5.2	100.
	2.	1-8.	2	4	4	4	4	3.6	100.
	3.	1-16.	1	1	2	2	2	1.6	80.
	4.	1-32.	1	0	2	0	0	0.6	30.
	5.	1-64.	0	0	0	0	0	0.0	20.
	6.	1-128.	0	0	0	0	0	0.0	20.
	7.	1-256.	0	0	0	0	0	0.0	0.
	8.	1-512.	0	0	0	0	0	0.0	0.
	9	1-1024.	0	0	0	0	0	0.0	0.

APPENDIX O

T. gondii strain RH. Details of time-to-death for mice in respect of each dilution of toxoplasma suspension.

Exp. # 1		Number of Mice		Dead on Day #										
Serial Two-Fold Dilution Number	Day 7	8	9	10	11	12	13	14	15	16	17	18	19	20
1		2	6	2										
2			5	2										
3			6	1										
4			3											
5			2	1										
6														
Expt. # 2														
2		4	5	1										
3		3	4	1										
4			3											
5			1	1										
6			2											
7														

APPENDIX P.

T. gondii strain V16 - Details of plaque numbers obtained and percentage of mice which died in respect of each dilution of toxoplasma suspension.

Experiment Number.	Serial Dilution Number.	Dilution Factor.	Plate #1.	Plate #2.	Plate #3.	Plate #4.	Plate #5.	Average per plate.	Per cent Mice Dead.
1.	A.	Undil.	166	144	161	152	147	154.0	N.D.
	B.	1-2.	84	71	70	78	68	74.2	N.D.
	C.	1-4.	39	39	45	35	37	39.0	N.D.
	D.	1-8.	12	21	23	21	17	18.8	100.
	1.	1-16.	12	10	12	12	14	12.0	90.
	2.	1-32.	10	5	4	6	5	6.0	100.
	3.	1-64.	7	3	2	1	2	3.0	90.
	4.	1-128.	3	1	1	0	2	1.4	70.
	5.	1-256.	1	0	0	1	1	0.6	50.
	6.	1-512.	1	0	0	0	0	0.2	20.
	7.	1-1024.	0	0	0	0	0	0.0	0.
	8.	1-2048.	0	0	0	0	0	0.2	0.
2.	A.	Undil.	85	87	94	102	87	91.0	N.D.
	B.	1-2.	45	48	50	51	46	48.0	N.D.
	1.	1-4.	20	19	18	21	17	19.0	100.
	2.	1-8.	9	9	8	8	10	8.8	100.
	3.	1-16.	5	5	4	6	4	4.8	90.
	4.	1-32.	2	3	2	2	3	2.4	90.
	5.	1-64.	1	1	3	1	2	1.6	80.
	6.	1-128.	1	1	1	1	1	1.0	60.
	7.	1-256.	1	0	2	0	0	0.6	50.
	8.	1-512.	0	1	1	0	0	0.4	20.
	9.	1-1024.	0	0	0	0	0	0.0	0.

APPENDIX Q

T. gondii strain V16. Details of time-to-death

Expt. # 1	Number of Mice Dead on Day # Day 7	8	9	10	11	12	13	14	15	16	17	18
Serial Two-fold Dilution D		2	1	4	3							
1			2	3	4							
2				7	2		1					
3				2	3	4						
4				3	2	2						
5				2	2			1				
6				1	1							
7												

Expt. # 2	Day 7	8	9	10	11	12	13	14	15	16	17	18
2		1	5	2	2							
3			2	4	1	2						
4				5	2	1	1					
5				4	4							
6				3	3							
7				1	3	1						
8					1	1						

APPENDIX R

T. gondii strain C56 - Details of plaque numbers obtained and percentage of mice which died in respect of each dilution of toxoplasma suspension.

Expt. No	Serial Dilution Number.	Dilution Factor.	Plate#1.	Plate#2.	Plate#3.	Plate#4.	Plate#5.	Average per plate.	Per cent Mice Dead.
1.	A	Undil.	123	119	113	113	110	115.6	N.D.
	B	1-2.	56	62	59	58	58	58.6	N.D.
	C	1-4.	26	29	30	31	30	29.2	N.D.
	1.	1-8.	16	16	17	15	x	16.0	100.
	2.	1-16.	7	7	8	x	9	7.75	100.
	3.	1-32.	5	6	5	5	4	5.0	80.
	4.	1-64.	2	2	2	2	4	2.4	60.
	5.	1-128.	2	1	1	0	1	1.0	60.
	6.	1-256.	1	0	0	1	0	0.4	20.
	7.	1-512.	0	2	0	0	0	0.4	20.
	8.	1-1024.	0	0	0	1	0	0.2	10.
	9.	1-2048.	0	0	0	0	0	0.0	0.
	10.	1-4096.	0	0	0	0	0	0.0	0.
2.	A	Undil.	27	28	30	28	29	28.4	N.D.
	B.	1-2.	12	12	14	11	13	12.4	N.D.
	C.	1-4.	8	8	8	9	5	7.6	N.D.
	1.	1-8.	5	4	4	2	x	3.75	90.
	2.	1-16.	2	2	2	1	x	1.75	90.
	3.	1-32.	1	1	1	3	1	1.4	60.
	4.	1-64.	1	0	0	0	1	0.4	30.
	5.	1-128.	0	0	1	0	0	0.2	10.
	6.	1-256.	0	0	0	0	0	0.0	10.
	7.	1-512.	0	o	0	0	0	0.0	0.
	8.	1-1024.	0	0	0	0	0	0.0	0.
	9.	1-2048.	0	0	0	0	0	0.0	0.
	10.	1-4096.	0	0	0	0	0	0.0	0.

APPENDIX S

T. gondii strain C56. Details of time-to-death

Expt. #	Serial Two-fold Dilution	Number of Mice Dead on Day # Day 7	8	9	10	11	12	13	14	15
1	2			1	4	2	2	1		
	3			1	2	3	2			
	4				1	3	2			
	5				1	3	1	1		
	6					1	1			
	7					1	1			
	8					1				
	9									
2	1			3	3	1	2			
	2			2	5	1	1			
	3			1	4		1			
	4				2			1		
	5					1				
	6				1					
	7									

APPENDIX T

T. gondii strain C37 - Details of plaque numbers obtained and percentage of mice which died in respect of each dilution of toxoplasma suspension.

Experiment Number.	Serial Dilution Number.	Dilution Factor.	Plate #1.	Plate #2.	Plate #3.	Plate #4.	Plate #5.	Average per plate.	Per cent Mice Dead.
1.	A.	Undil.	112	108	102	113	x	109.0	N.D.
	B.	1-2.	54	55	52	58	52	54.2	N.D.
	C.	1-4.	28	27	30	30	31	29.2	N.D.
	1.	1-8.	17	16	17	18	16	16.8	100.
	2.	1-16.	9	9	10	8	10	9.2	90.
	3.	1-32.	5	6	5	6	5	5.4	80.
	4.	1-64.	2	3	3	3	2	2.6	50.
	5.	1-128.	1	1	1	1	1	1.0	20.
	6.	1-256.	0	0	1	0	0	0.2	0.
	7.	1-512.	0	0	0	0	0	0.0	10.
	8.	1-1024.	0	0	0	0	0	0.0	10.
	9.	1-2048.	0	0	0	0	0	0.0	0.
	10.	1-4096.	0	0	0	0	0	0.0	0.
2.	A.	Undil.	36	31	32	34	31	32.8	N.D.
	B.	1-2.	17	17	16	16	15	16.2	N.D.
	1.	1-4.	11	9	8	9	9	9.2	100.
	2.	1-8.	5	4	4	4	4	4.2	60.
	3.	1-16.	3	2	2	3	3	2.6	50.
	4.	1-32.	1	1	1	1	1	1.0	30.
	5.	1-64.	0	1	0	0	1	0.4	20.
	6.	1-128.	0	0	0	0	1	0.2	20.
	7.	1-256.	0	0	0	0	0	0.0	0.
	8.	1-512.	0	0	0	0	0	0.0	0.
	9.	1-1024.	0	0	0	0	0	0.0	0.

APPENDIX U

T. gondii strain C37. Details of time-to-death

Number of Mice Dead on Day #

Expt. #	Serial Two-fold Dilution	Day 7	8	9	10	11	12	13	14	15
1										
	1				3	6	1			
	2					1	3	4	1	
	3					2	4	2		
	4						2	3		
	5						1	1		
	6									
	7									1
	8									1
	9									
2	1				1	5	3	1		
	2						2	3	1	
	3					2	2			1
	4					1	2			
	5					1	1			
	6						1	1		

APPENDIX V.

T. gondii strain Beverley - Details of plaque numbers obtained and percentage of mice which died in respect of each dilution of toxoplasma suspension.

Experiment Number.	Serial Dilution Number.	Dilution Factor.	Plate #1.	Plate #2.	Plate #3.	Plate #4.	Plate #5.	Average per plate.	Per cent Mice Dead.
1.	A.	Undil.	50	50	49	49	48	49.2	N.D.
	B.	1-2.	22	22	24	24	24	23.2	N.D.
	1.	1-4.	9	10	10	13	11	10.6	100.
	2.	1-8.	6	7	6	6	5	6.0	100.
	3.	1-16.	3	3	4	4	4	3.6	90.
	4.	1-32.	2	2	1	3	2	2.0	60.
	5.	1-64.	0	1	0	2	1	0.8	30.
	6.	1-128.	2	0	0	0	2	0.8	20.
	7.	1-256.	0	0	1	1	0	0.4	10.
	8.	1-512.	0	0	0	0	0	0.0	0.
	9.	1-1024.	0	0	0	0	0	0.0	0.
2.	A.	Undil.	112	116	113	110	112	112.6	N.D.
	B.	1-2.	56	60	52	54	56	55.6	N.D.
	1.	1-4.	26	24	26	22	28	25.2	100.
	2.	1-8.	12	15	14	15	13	13.8	100.
	3.	1-16.	7	6	6	7	7	6.8	100.
	4.	1-32.	4	3	3	2	2	2.8	55.5.
	5.	1-64.	2	1	2	1	2	1.6	20.0.
	6.	1-128.	1	1	0	1	1	0.8	20.0.
	7.	1-256.	0	0	0	0	0	0.0	10.
	8.	1-512.	0	0	0	0	0	0.0	0.

APPENDIX W

T. gondii strain Beverley. Details of time-to-death

Number of Mice Dead on Day #

Expt. #	Serial Two-fold Dilution	Day 7	8	9	10	11	12	13	14	15	16	17
1	2				5	2	1	2				
	3				1	2	5	1				
	4				2	2	1	1				
	5				2	1						
	6						2					
	7									1		
	8											
2	2					1	3	3		3		
	3					2	3	1	1	2	1	
	4					1		2	2			
	5						1		1			
	6							2				
	7							1				
	8											