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I hereby recommend that the thesis prepared under my supervision by John O. MacFarlane

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THE CULTIVATION OF TOXOPLASMA IN DEVELOPING CHICK EMBRYOS AND THE USE OF INFECTED EMBRYONAL TISSUES IN THE COMPLEMENT FIXATION TEST

A dissertation submitted to the

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by

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J.O.M.

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Reports of the cultivation of toxoplasma in the developing chick embryo have appeared in the literature but these accounts are brief and give little experimental data. Levaditi, et al, (1) and Wolfe, Cowen, and Paige (2) stated that inoculated fertile eggs hatched but that the chicks died within several days and their tissues were parasitized. Weinman (3) found that numerous small white nodules developed on the chorio-allantoic membrane after chorio-allantoic or yolk sac inoculation. In the above investigations recently isolated strains of toxoplasma were used for egg inoculation; deaths appeared to have been infrequent for they were not mentioned specifically. Inoculation of eight- and ten-day old duck embryos with avian strains of toxoplasma was reported by Wolfson (4).

Successful cultivation of toxoplasma in tissue cultures has been reported by several investigators (5), (6), (7).

In addition to the cultivation of viruses, rickettsiae, and bacteria, many protozoa have been successfully grown in developing hen's eggs. Burnet and Beveridge (8) have published a monograph giving the various procedures and uses to which the embryonated egg has been applied.

The data to be presented demonstrate that a strain of human origin, well adapted to mice, not only readily infected eggs but also produced such uniform embryo deaths as to make

the embryonated egg a satisfactory titration medium. Another strain of human origin which gave irregular results in mice also produced embryo deaths regularly and behaved in a manner similar to that of the strain well adapted to mice.

The serum neutralization test as described by Sabin and Ruchman (9), (10) is the procedure generally employed for detection of antibodies to toxoplasma. In this test equal quantities of undiluted serum are mixed with various dilutions of virulent toxoplasma and the mixtures injected intracutaneously in the rabbit's back. Results are not obtained until after approximately seven days. Nicolau and Ravelo (11) had reported positive complement fixation reactions with the sera and extracts of various tissues of experimentally infected animals. A methyl alcohol extract of desiccated spleen was their antigen of choice. Warren and Sabin (12) also investigated the complement fixation reaction in toxoplasma infection. They were unable to repeat the results of Nicolau and Ravelo with a rabbit spleen extract, but did obtain specific complement fixation of immune monkey sera with frozen and thawed saline extracts of infected rabbit brain. In order to detect specific fixation it was necessary for them to test the sera simultaneously against an extract of normal rabbit brain and the extract of infected rabbit brain.

Attempts by the author to repeat both the work of Nicolau

and Ravelo and of Warren and Sabin failed; therefore with the adaptation of toxoplasma to growth in the tissues of the embryonated egg, a new source of antigenic material became available. This communication will describe the use of such antigens in the complement fixation reaction. Complement fixation tests have been performed with sera of rhesus monkeys, rats, pigeons, rabbits, chicks, guinea pigs, and human beings.

MATERIALS and METHODS

Strains of toxoplasma: The R.H. strain of toxoplasma, isolated by Sabin (13) from a fatal human case of encephalitis, was used in most of these studies. It was maintained in this laboratory by continuous intracerebral passages in mice, and the 304th to 395th passage material was used for initiating the cultures in embryonated eggs. This R.H. strain was well adapted to mice, and intracerebral or intraperitoneal inoculation always resulted in death. On several occasions the C.G. strain of toxoplasma, isolated by Johansmann and Ruchman (14) from an infant who died of toxoplasmosis, was used for the initiation of embryonic egg passages and for the production of immune sera. This C.G. strain was also maintained by intracerebral mouse passage in our laboratory, and material from the 9th to 14th mouse passages was used; however this strain when injected into mice only produced an occasional death. Brains

from sacrificed mice showed numerous toxoplasma, and surviving mice were immune to subsequent challenge with the R.H. strain.

Mice: Albino mice, bred in our own laboratory and weighing approximately 15 to 22 grams, were injected intracerebrally with 0.03 ml of the test suspension unless otherwise specified. Intracerebral injection of 10 per cent infected mouse brain suspensions of the R.H. strain resulted in deaths between the 3rd and 6th days and gave LD 50 titers between $10^{-4.0}$ and $10^{-5.0}$.

Chick embryos: Fertile hen's eggs from pullorum-tested flocks were used, and incubation was at 37 C both before and after inoculation with but one exception. Two methods of inoculation were utilized, as follows:

Yolk sac method: After washing the air-sac end of the egg first with 70 per cent alcohol and then with tincture of iodine, a hole was drilled and the inoculation was performed through this hole by a syringe equipped with a 22-gauge needle.

Chorio-allantoic membrane method: By means of an alundum disc on a power drill a triangular piece of egg shell was cut from the side of the egg without damaging the egg shell membrane. After this triangle had been removed with sterile forceps, a drop of sterile saline was applied to the exposed egg shell membrane and a slit made in the shell membrane with a needle. By applying slight suction from a rubber bulb to a hole drilled in the air sac end of the egg, an artificial air

sac was created at the site of the triangular opening. The inoculum was then dropped on the exposed chorio-allantoic membrane. The hole was sealed with "scotch tape" and the egg incubated on its side.

Eggs were candled once or twice daily. All embryos that died within the first three days were found to have died from non-specific causes such as trauma and bacterial contaminants, and were therefore discarded. Membranes and tissues were harvested as soon as possible after death or were kept at 4 C until required. Presence or absence of toxoplasma was determined by smears stained with Wright's stain or by mouse inoculation.

Preparation of inoculum: Inocula were saline suspensions of infected tissues prepared by (1) grinding in a mortar, (2) blending in a Waring blendor, or (3) shaking the tissues with glass beads in a 250 ml centrifuge bottle. The latter method was utilized most frequently.

Calculation of Titers: LD 50 titers in mice and chick embryos were calculated according to the method of Reed and Muench (15).

EXPERIMENTAL

Injection of toxoplasma-infected mouse brain suspensions

into the yolk sac of embryonated eggs on at least nine different occasions led to multiplication of the parasites with resulting death of the embryo in 4 to 10 days after inoculation. Examination of dead embryos revealed the presence of numerous yellow-white plaques, 0.5 to 3 mm in diameter, on the chorio-allantoic and amniotic membranes (Fig. 1). The areas surrounding the lesions were thickened and histologically represented regions of dense cellular infiltration containing numerous toxoplasma (Fig. 2). Higher magnification (1200x) of an area of cellular infiltration revealed toxoplasma in both crescentic and rounded forms (Fig. 3). Smears of the chorio-allantoic membrane and yolk sac, stained with Wright's stain, revealed numerous toxoplasma both free and intracellular. Cross (16) has suggested the designation "terminal colony" for these aggregates of intracellular toxoplasma which are generally termed "pseudocysts".

Table 1 illustrates the relative ease with which embryos are infected upon initial egg passage using the R.H. strain of toxoplasma. All embryos inoculated with 320 or more LD 50 mouse doses, two of three eggs injected with 32 LD 50 doses, and one of three injected with 3 LD 50 doses died. Similar results were obtained in two additional experiments. Six other attempts were made to institute egg passages. The inoculation of 0.5 ml of a 10 per cent mouse brain suspension

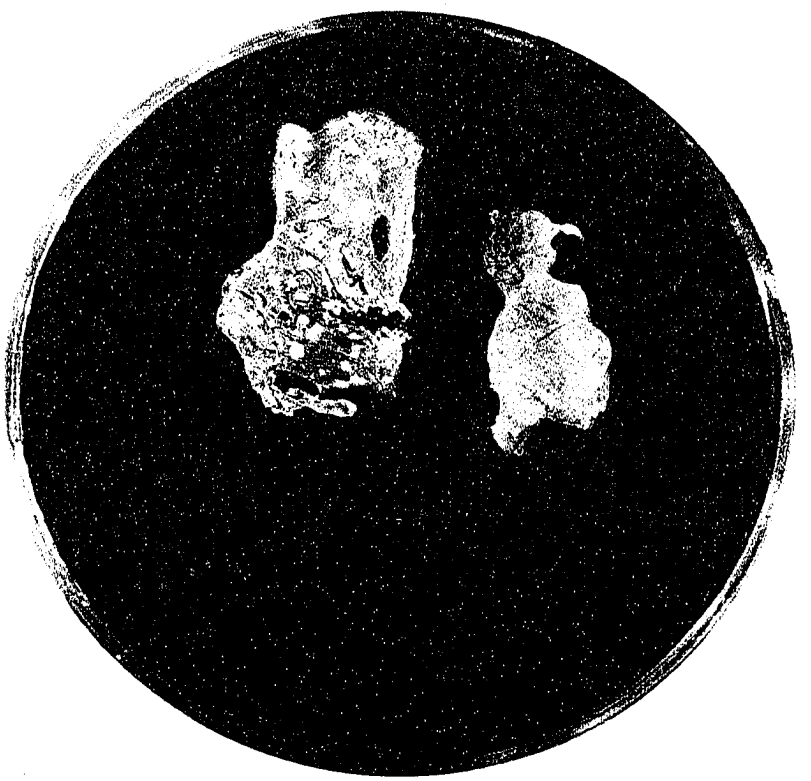


Fig. 1.

Toxoplasma in the developing chick embryo
Eggs 18 days old

Left. Infected chorio-allantoic membrane
Right. Normal chorio-allantoic membrane

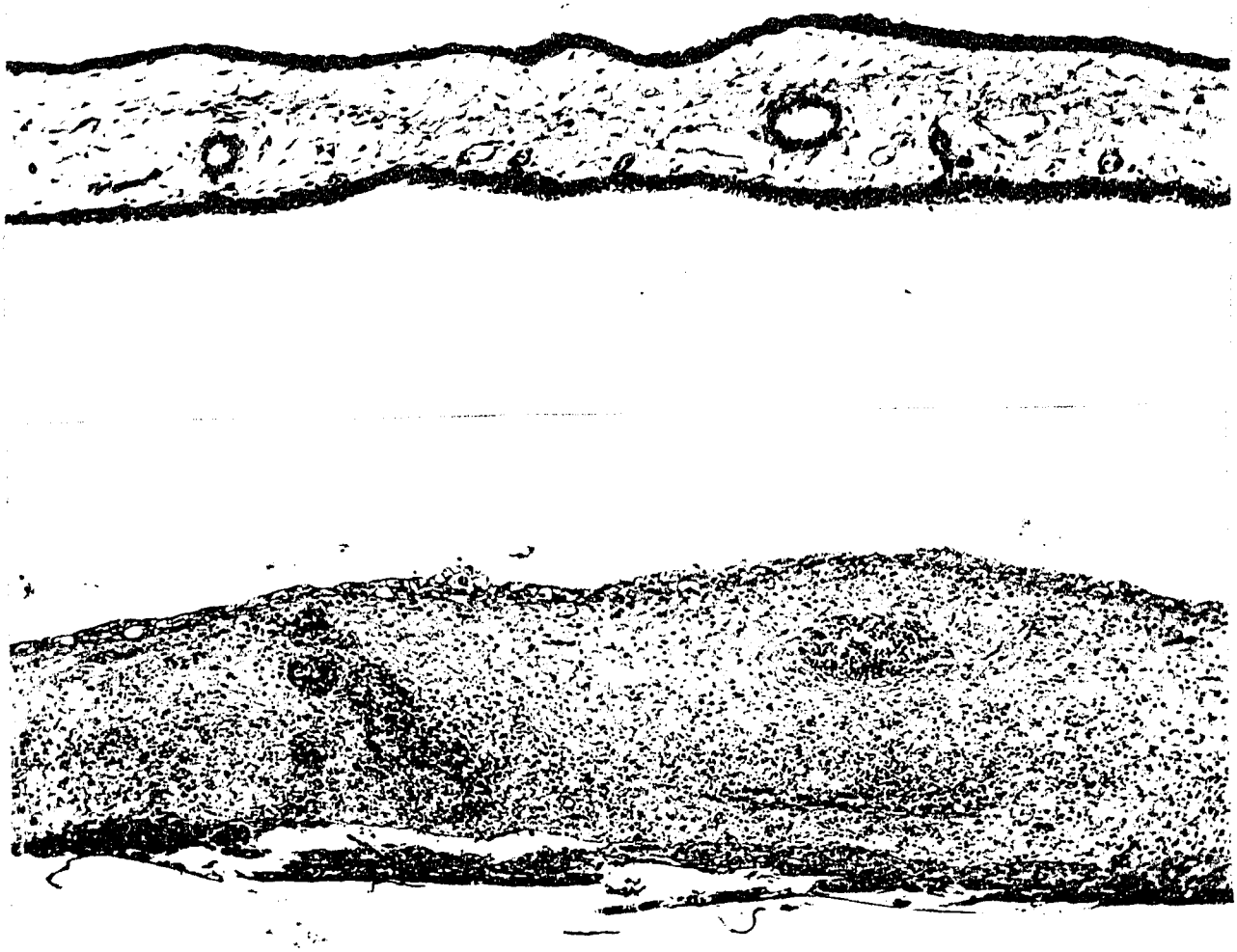


Fig. 2.

Toxoplasma in the developing chick embryo
Eggs 18 days old. H. and E. x160

Upper. Normal chorio-allantoic membrane
Lower. Infected chorio-allantoic membrane

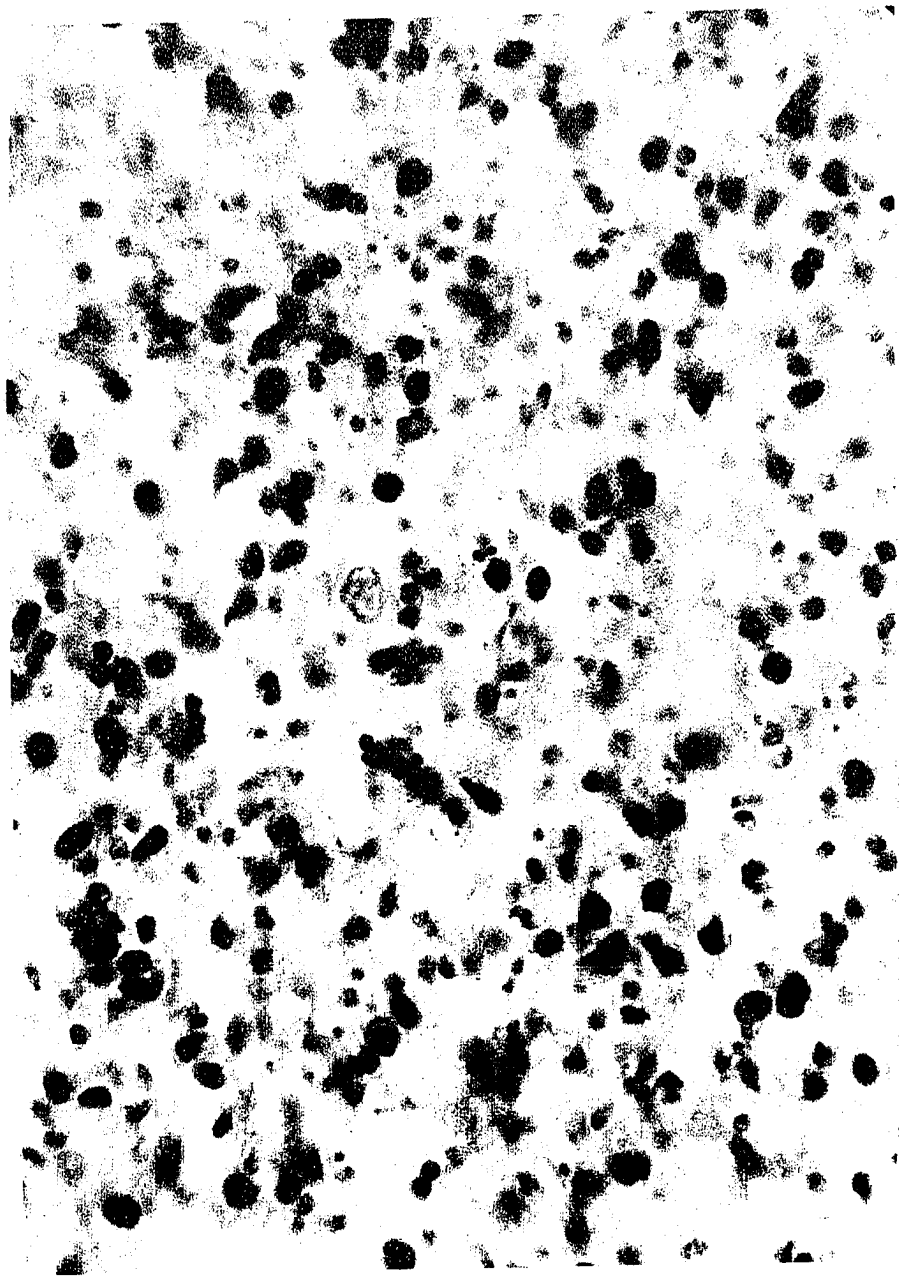


Fig. 3.

Toxoplasma in the developing chick embryo
Eggs 18 days old. H. and E. x1200

Chorio-allantoic Membrane

Table 1

Initial Chick Embryo Inoculations
with Mouse Brain Suspension

10-day embryos inoculated with 0.5 ml via yolk sac

Dilution of Mouse Brain	Mouse LD_{50} Doses* Per Embryo	Days of Specific Deaths
10^{-1}	32000	6,7
10^{-2}	3200	6,7,7
10^{-3}	320	7,8,8
10^{-4}	32	9,9,H**
10^{-5}	3	9,H,H
10^{-6}	0.3	H,H

* These LD_{50} titers were determined by intraperitoneal injection of 0.5 ml into mice.

** H = egg hatched.

per egg was sufficient to cause the death of all embryos by the 8th day after inoculation. Only embryos inoculated with dilutions of 10^{-4}, 10^{-5}, 10^{-6} hatched, and none of the chicks which hatched were shown to contain toxoplasma by Wright's smear and mouse inoculation; however the tissues from all chicks were not sub-inoculated into mice.

Using the R.H. strain of toxoplasma, four series of egg passages were studied in order to seek any evidence of adaptation or change. One series was carried through twenty-seven consecutive passages in ten- and eleven-day fertile eggs (Table 2a). A second series was carried through 11 serial passages in ten-day chick embryos (Table 2b). A third series (Table 2c, egg passages 1 S.B. to 5 S.B.) was made in nine- and ten-day incubated eggs, and three serial egg passages were made in which six- and seven-day incubated eggs were utilized (Table 2c, egg passages 1 S.C. to 3 S.C.).

No evidence was obtained that continuous or multiple egg passages altered the length of time between inoculation and death of the embryo. Regardless of the number of egg passages the embryos generally died between the 4th and 8th days after inoculation of 10 per cent mouse brain suspension or suspension of a pool of yolk sac and chorio-allantoic membrane. Most of the deaths occurred on the 5th and 6th days. As is shown in Table 1, inoculation of higher dilutions

Table 2a

Toxoplasma Passages in Eggs Inoculated via the Yolk Sac

Egg Passage	Age of Eggs	Tissue Suspension Employed	Am't	Day of Death	LD_{50} Titers in Mice		
					Yolk Sac	C.A.	Pool of Y.S.-C.A.
	days		ml				
1*	11	10% Mouse Brain	0.25	5,5,6,6,6,6,7,7,7,7	$10^{-3.5}$	$10^{-4.0}$	
4*	11	5% Y.S.-C.A.	0.5	6,6,6,6,6			$10^{-4.5}$
7	10	10% "	"	5,6,6,6			$10^{-5.5}$
10	10	" "	"	4,5,6,6,6,6,6,6,6,6			$10^{-5.0}$
12	10	" "	"	4,4,5,5,5,5,5,5			$10^{-4.5}$
15**	10	" "	"	6,6,6,10			$10^{-4.0}$
20	10	" "	"	5,5,5,5,6,6,6,6			$10^{-5.0}$
27	10	" "	"	6,6,6,6,6	$10^{-3.5}$	$10^{-4.5}$	

* Inocula for these groups were ground with mortar and pestle. All other inocula were macerated by shaking with glass beads.

** Inoculated with membranes which had been stored 5 days at 4 C.

Table 2b

Toxoplasma Passages in Eggs Inoculated via the Yolk Sac

Egg Passage	Age of Eggs	Tissue Suspension Employed	Am't	Day of Death	LD_{50} Titers in Mice			
					Yolk Sac	C.A.	Whole Embryos	Pooled Embryonic Fluids
	days		ml					
1-48	10	5% Mouse Brain	0.5	6,6,6,6,7,7,7,7				
2-48	10	10% Y.S.	0.5	5,5,5,6,6,6,6				
3-48	10	20% Y.S.-C.A.	0.25	5 (19 embryos)		$10^{-4.7}$		
4-48*	10	20% Y.S.	0.5	6,6,7,7,8				
5-48	10	20% Y.S.-C.A.	0.5	4 (4 embryos) 5 (3 ") 6 (18 ") 7 (2 ")				
6-48	10	50% Y.S.-C.A.	0.05	5,5,5,5,6,6,6,6,6,6,6,6				
7-48	11	20% Y.S.	0.4	4,5,6,6,6,6,7	$10^{-4.5}$	$10^{-5.0}$	$>10^{-4.0}$	$10^{-4.0}$
8-48	10	10% "	0.5	6,6,6,6,6,6,6,6,6,6,7,7				
9-48	10	10% Y.S.-C.A.	0.5	5,6,6,6,6,6,6,7,7,7				
10-48	10	20% "	0.5	5,5,5				
11-48**	11	" "	0.5	6,6,7,8,8,8	$10^{-5.0}$	$10^{-4.5}$	$10^{-4.5}$	$10^{-2.5}$

* Inoculated with membranes which had been stored 7 days at 4 C.
** " " " " " " " overnight " " .

Table 2c

Toxoplasma Passages in Eggs Inoculated via the Yolk Sac

Egg Passage	Age of Eggs	Tissue Suspension Employed	Am't	Day of Death	LD_{50} Titers in Mice of Pool of Y.S.-C.A.
	days		ml		
1 SB	9	10% Mouse Brain	0.5	5,7,7	$10^{-4.0}$
2 SB	9	10% Y.S.-C.A.	"	6,6,6,6	$10^{-4.0}$
3 SB	10	" "	"	6,6	$10^{-4.5}$
4 SB	10	" "	"	4,5,5,5	$10^{-5.0}$
5 SB	10	" "	"	5,5,6,6	
1 SC*	7	10% Mouse Brain	0.25	4,5,6,8	
2 SC*	6	10% Y.S.-C.A.	"	4,6,7,7	
3 SC*	7	20% "	"	4,5,5,6	

* Inocula for these groups ground with mortar and pestle.

All other inocula macerated by shaking with glass beads.

lengthened the survival period, and deaths occurred on the ninth day for eggs inoculated with 10^{-4} and 10^{-5} dilutions. The days of death after inoculation of six- and seven-day chick embryos appear to be spread over a slightly longer period.

Titrations in mice were performed utilizing yolk sac (Y.S.), chorio-allantoic membrane (C.A.) or a pool of the yolk sac and chorio-allantoic membrane (Y.S.-C.A.). These titrations were made by intracerebral injections of 0.03 ml of 10-fold dilutions, each dilution being injected into 2 or more mice. No obvious change in titer was obtained after 27 egg passages; the yolk sac titering $10^{-3.5}$ at the 1st and 27th passages, and the C.A. titering $10^{-4.0}$ at the 1st passage and $10^{-4.5}$ at the 27th passage. The titers of the combined Y.S.-C.A. in between the 1st and 27th passages ranged from $10^{-4.0}$ to $10^{-5.5}$ with this one instance of a $10^{-5.5}$ titer being one of the only two titers over $10^{-5.0}$ encountered during these experiments. All titers of the series in nine- and ten-day eggs were in the range of $10^{-4.0}$ to $10^{-5.0}$. These were approximately the same titers usually attained in mice.

On two occasions the C.G. strain was used to initiate egg passages, and on both occasions the embryos were dead on the 7th, 8th, and 9th days after yolk sac inoculation of

ten-day embryos with 10 per cent mouse brain (Table 2d). Four mice inoculated intracerebrally with the same 10^{-1} mouse brain suspension used to initiate egg passage 1 (C.G. strain from 9th mouse passage) survived. Of five mice inoculated with the same 10 per cent mouse brain suspension used to initiate egg passage 1B (C.G. strain from 14th mouse passage) two mice were sick and sacrificed on the 5th day, one died on the 9th day, and two survived. This strain required one or two days longer to kill chick embryos than did the R.H. strain, and this characteristic of the C.G. strain persisted for six serial passages during which the bulk of embryo deaths occurred on the 6th and 7th days (Table 2d). Otherwise the C.G. strain behaved in chick embryos as did the R.H. strain.

As previously mentioned, Wright's stained smears revealed that the largest number of parasites were located in the yolk sac and C.A. membrane, with occasional organisms present in the fluids, lungs, brain, and viscera of the embryo. In order to verify this distribution simultaneous titrations of various egg tissues from the same eggs were performed in mice and the LD 50 titers are recorded in table 3. The chorio-allantoic membranes contained the greatest number of parasites, but gave only slightly higher titers than the yolk sacs. LD 50 titers for first egg passage tissues were $10^{-4.0}$ for the C.A. and $10^{-3.5}$ for the yolk sac. Twenty-seventh egg passage materials had titers of $10^{-4.5}$, $10^{-3.5}$, $10^{-3.0}$

Table 2d

Toxoplasma Passages in Eggs Inoculated via the Yolk Sac with C.G. Strain

Egg Passage	Age of Eggs	Tissue Suspension Employed	Am't	Day of Death
	days		ml	
1*	10	10^{-1} Mouse Brain	0.5	7,7,8,9
		10^{-2} " "	"	8,9,9,9,9
		10^{-3} " "	"	8,9,9,9,9,10
		10^{-4} " "	"	9,9,L,L,L,L**
2	10	20% Y.S.-C.A.	"	5,6,7,7,7
3	10	" "	"	6,6,6,7,7,7
4	11	" "	"	6,6,7
5	10	10% "	"	6,7,7,7
6	10	" "	"	4,6,6,6
1B***	10	10% Mouse Brain	"	7,8,8,8,9,9,9,9

* Four mice inoculated with same 10^{-1} mouse brain suspension survived. (C.G. passage 9.)

** Four embryos living at end of tenth day were ready to hatch.

*** Of five mice inoculated with same 10% mouse brain, two were sacrificed on the fifth day, one died on the ninth day, and two survived. (C.G. passage 14.)

Table 3

Distribution of Toxoplasma in Eggs Inoculated via the Yolk Sac with R.H. Strain

Egg Passage	Inoculation		Number of Eggs	LD_{50} Titer in Mice			
	Tissue	Am't		Yolk Sac	C.A.	Embryonic Tissues	Pooled Embryonic Fluids
		ml					
1*	10% Mouse Brain	0.25	4	$10^{-3.5}$	$10^{-4.0}$		
27*	10% Y.S.-C.A.	0.5	2	$10^{-3.5}$	$10^{-4.5}$	$<10^{-2.0}$	$10^{-3.0}$
7-48**	20% Y.S.	"	3	$10^{-4.5}$	$10^{-5.0}$	$>10^{-4.5}$***	$10^{-4.0}$
11-48**	20% Y.S.-C.A.	"	2	$10^{-5.0}$	$10^{-4.5}$	$10^{-4.5}$***	$10^{-2.5}$

* Tissues macerated by grinding in mortars.
** " " " blending " Waring blendor.
*** Whole embryos macerated.

and less than $10^{-2.0}$ for the C.A. membranes, yolk sacs, pooled embryonic fluids, and tissues (brain, lungs, heart, liver, and kidneys) of the embryos, respectively. In these two comparative titrations the tissues were macerated by grinding in a mortar. Two later titrations, with the tissues of egg passages 7 and 11 of the 1948 series, were performed in mice using tissues macerated by blending in Waring blendors. This was done by placing the tissues and saline diluent in the blendor and blending for two minutes. In these two instances the whole embryos were titrated instead of only the viscera, as was done for egg passages 1 and 27. The titers thus obtained were slightly higher, and the LD 50 titers for the whole embryos, $> 10^{-4.5}$ and $10^{-4.5}$, were similar to those obtained with the C.A. membranes and the yolk sacs. The pooled embryonic fluids in all cases gave the lowest LD 50 titers, namely $10^{-3.0}$, $10^{-4.0}$, and $10^{-2.5}$. These lower values of the fluids are in accord with the intracellular nature of toxoplasma.

After six serial passages through chick embryos, titrations in mice were made in order to determine the distribution of parasites in the various tissues (Table 4) of dead embryos after injection with the C.G. strain of toxoplasma. Material from three embryos dead six days after injection were blended in the manner previously described.

Table 4

Distribution of Toxoplasma in Eggs Inoculated via the Yolk Sac with C.G. Strain

3 eggs from egg passage 6

Tissue	LD_{50} Titer in Mice	Titer Corrected* for Immune Survivors
Yolk Sac	$10^{-3.0}$	$10^{-3.5}$
Chorio-allantoic Membrane	$10^{-3.5}$	$10^{-5.0}$
Whole Embryos	$10^{-2.4}$	$10^{-4.5}$
Pooled Embryonic Fluids	$10^{-3.0}$	$10^{-3.0}$

* This titer was obtained by intracerebral challenge of mice surviving LD_{50} titration with 0.03 ml of 10% C.G. strain infected mouse brain.

Deaths occurred in the titration mice between the tenth and 18th days. The LD 50 titers were: yolk sac, $10^{-3.0}$; chorio-allantoic membrane, $10^{-3.5}$; whole embryos, $10^{-2.4}$; and the pooled embryonic fluids, $10^{-3.0}$. Some mice other than those that died appeared sick but survived; therefore twenty-seven days after the original inoculation these mice were challenged intracerebrally with 0.03 ml of 10 per cent mouse brain from C.G. infected mice to determine the number of immune mice. When the numbers of immune mice were added to the original LD 50 titers, the corrected titers were: yolk sac, $10^{-3.5}$; chorio-allantoic membrane, $10^{-5.0}$; whole embryos, $10^{-4.5}$; and pooled embryonic fluids, $10^{-3.0}$. These titers are in close agreement with the titers obtained in chick embryos infected with the R.H. strain.

Comparative titrations in mice were made with the membranes harvested from dead and living chick embryos of a group inoculated with the same suspension (Table 5). Such titrations were performed for eggs dead or living before the maximum day of death and for dead and living eggs on the maximum day of death. The maximum day of death was that day on which the greatest number of embryos died. Titration of egg passage 12 material gave LD 50 titers of $10^{-4.5}$ for membranes of eggs dead and living 5 days after inoculation, and the same titer for membranes of eggs dead on the 5th day which was the day on which

Table 5

Comparative Titrations in Mice of Membranes Harvested from Dead and Living Chick Embryos

10-day embryos inoculated with 0.5 ml of Y.S.-C.A. Susp. via yolk sac

Egg Passage	Maximum Days of Embryo Deaths	LD_{50} Titers					
		Number of Days after Egg Inoculation					
		4		5		6	
		Dead	Living	Dead	Living	Dead	Living
12	5	$10^{-4.5}$	$10^{-4.5}$	$10^{-4.5}$			
16	6		$10^{-3.5}$		$10^{-5.0}$	$<10^{-0.5}$*	$10^{-4.5}$
20	5,6		$10^{-3.5}$	$10^{-5.0}$	$10^{-5.0}$	$10^{-5.0}$	

* Mice were inoculated with Y,S.-C.A. from eggs dead 6 days after inoculation of sixteenth egg passage; however, something completely inactivated original 10% Y.S.-C.A. suspension.

the maximum number of embryo deaths occurred; thus the titers were equally as high for membranes of eggs dead or living one day before the day on which the greatest number of embryo deaths occurred as for the membranes of eggs dead on the maximum day of death. Data from egg passage 16 also indicated that this was true, but that membranes from eggs dead two days before the maximum day of death had somewhat lower LD 50 titers, $10^{-3.5}$ as compared with $10^{-5.0}$ and $10^{-4.5}$. Data from egg passage 20 were more difficult to interpret since the maximum deaths were equally divided between the 5th and 6th days; however the titers of the membranes, $10^{-5.0}$, were identical for eggs dead 5 days, living 5 days, and dead 6 days after inoculation. In this instance the membranes of eggs harvested earlier, that is, 4 days after inoculation had a lower titer, namely $10^{-3.5}$. Thus it appeared that membranes harvested either from dead or living chick embryos at or near the time of the maximum number of embryo deaths possessed similar high titers and that membranes of living eggs harvested two days before this maximum had somewhat lower titers.

Four comparative titrations were performed in mice and chick embryos, and the results are listed in table 6. In three cases mice were inoculated intracerebrally with 0.03 ml, and chick embryos were inoculated into the yolk sac with 0.5 ml of the test suspension. Infected mouse brain suspensions were titered in three tests, and a yolk sac-chorio-

Table 6

Comparative Titrations of Toxoplasma Infected Tissues in Mice and Developing Chick Embryos

10-day chick embryos inoculated via the yolk sac
Mice inoculated intracerebrally

Passage	Inoculation			LD_{50} Titer	LD_{50} Doses per Gram of Indicated Tissue
	Tissue	Am't	Titration in:		
		ml			
335	Mouse Brain	0.5	Embryos	$10^{-5.0+}$	200,000+
		0.03	Mice	$10^{-4.5}$	1,000,000
336	" "	0.5	Embryos	$10^{-5.0}$	200,000
		0.03	Mice	$10^{-4.0}$	330,000
344	" "	0.5	Embryos	$10^{-4.5}$	64,000
		0.5*	Mice	$10^{-4.5}$	64,000
15	Y.S.-C.A.	0.5	Embryos	$10^{-4.0}$	20,000
		0.03	Mice	$10^{-3.0}$	33,000

* Mice in this case were injected intraperitoneally.

allantoic membrane pool was used in the fourth series. In three of the tests, passages 335, 336, and 15, higher LD 50 titers were obtained in chick embryos than in mice. However, in three cases the amount injected into eggs was proportionately (16x) larger. The mouse brain suspension of passage 335 titered $10^{-5.0+}$ in embryos as against $10^{-4.5}$ in mice. Similarly, passage 336 titered $10^{-5.0}$ in eggs and $10^{-4.0}$ in mice, and a yolk sac-chorio-allantoic membrane suspension from egg passage 15 titered $10^{-4.0}$ in eggs and $10^{-3.0}$ in mice. When the actual number of LD 50 doses per gram of inoculated tissue were calculated, the values obtained in these three tests were slightly higher in mice than in chick embryos, 1,000,000 as against 200,000+, 330,000 as against 200,000 and 33,000 as against 20,000. The mouse brain suspension of passage 344 was titered with similar inocula, 0.5 ml per egg via the yolk sac and 0.5 ml per mouse intraperitoneally. Both LD 50 titers were $10^{-4.5}$, and the LD 50 doses per gram of titrated tissue were the same, 64,000 LD 50 doses.

Results in tables 2a, 2b, 2c, and 2d show that 6-, 7-, 9-, 10-, and 11-day fertile hen's eggs were used for the successful cultivation of toxoplasma. On three occasions direct comparisons were made between embryos of different age, and the results are listed in table 7. In one experiment in which seven- and eleven-day eggs were inoculated

Table 7

Influence of Age of Developing Chick Embryo Infected via the Yolk Sac

Egg Passage	Inoculation		Age of Eggs	Day of Death
	Tissue Suspension	Volume		
		ml	days	
1	10% Mouse Brain	0.25	7	4,5,6,8
			11	5,5,6,6,6,6,7,7,7,7
10	10% Y.S-C.A.	0.5	10	4,6,6,6,7
			11	4,5,6,6,6,6,6,6,6
7-48	20% Y.S.	0.4	7	4,5,5,5,5,6,6,6,6,6,6,7
			11	4,5,6,6,6,6,6,7

with the same 10 per cent mouse brain suspension, the seven-day eggs died between the 4th and 8th days, and the eleven-day eggs died between the 5th and 7th days. In another experiment all deaths in both seven- and eleven-day embryos which were inoculated with a 20 per cent yolk sac suspension fell between the 4th and 7th days. Ten- and eleven-day chick embryos inoculated simultaneously with 10 per cent Y.S.-C.A. material showed no differences.

With some disease agents which are cultivable in developing chick embryos, incubation of the inoculated eggs at temperatures below 37 C has been found to give more favorable results. The data in table 8 demonstrate that chick embryos incubated at 35 C survived an average of one day longer than chick embryos incubated at 37 C. All eggs succumbed at both temperatures; however, examination of the chorio-allantoic membranes revealed that the plaques in the eggs that were incubated at 35 C were generally of 0.5 mm size, whereas the plaques in eggs incubated at 37 C were of 1.0 to 2.0 mm size.

In these experiments inoculation of fertile eggs was usually into the yolk sac; however,, the chorio-allantoic membrane method of inoculation also produced embryo deaths. By comparative inoculation of ten-day chick embryos via the yolk sac and onto the chorio-allantoic membrane (Table 9) with equal quantities of the same inoculum,, the deaths of

Table 8

Influence of Temperature of
Incubation of Developing Chick Embryos
Infected via the Yolk Sac
with
0.5 ml 10% Y.S.-C.A. per egg in 10-day chick embryos

Temperature	Day of Death	Average Size of Plaques
°C		mm
37	5,6,6,6,6,6,6,7,7,7	1.0 - 2.0
35	6,7,7,7,7,7,8,8,8,8,8	0.5

Table 9

Influence of Method of Inoculation
of Developing Chick Embryos Infected
with
0.05 ml of 50% Y.S.-C.A. per egg in 10-day chick embryos

Method of Inoculation	Day of Death	Description of Plaques
Yolk Sac	5,5,5,5,6,6,6,6,6,6,6,6	Numerous, 1.0 - 2.0 mm, universally distributed over C.A.
Chorio-allantoic Membrane	4,5,5,5,5,6,6,6,6,6,6	Fair number, 0.5 - 1.5 mm, mainly concentrated at the site of inoculation on C.A.

the embryos were concentrated on the 5th and 6th days regardless of the method of introduction of the parasites. Plaques were present on the chorio-allantoic membranes of both sets of embryos, but their distributions varied. After inoculation onto the chorio-allantoic membranes, 0.5- to 1.5-mm plaques were found in fair numbers, and were concentrated mainly at the site of inoculation but were not confined to that area. Inoculation into the yolk sac produced numerous 1- to 2-mm plaques which were universally distributed over the entire chorio-allantoic membrane.

Chick embryo membranes, and saline suspensions of these tissues, were tested after various periods of storage for the presence of viable toxoplasma. At least two mice, and generally four mice, were injected with the test material. Each mouse received 0.03 ml intracerebrally, and 0.5 to 1.0 ml intraperitoneally, of a 10 or 20 per cent saline suspension of the stored material. These percentages were calculated on the basis of the original membrane weights. Membranes were harvested aseptically from the embryos, placed in sterile test tubes either as whole membranes or as saline suspensions, and the tubes were sealed with parafilm in order to prevent dehydration. Table 10 presents the data from two experiments in which whole yolk sacs and chorio-allantoic membranes were stored at 4 C. The yolk sac

and the chorio-allantoic membrane from the same egg were tested simultaneously in order to eliminate any egg to egg variation. With membranes of egg passage 19 viable toxoplasma were found in the yolk sac after 21 days but not after 36 days. The materials from egg passage 5-48 yielded viable parasites from both yolk sac and chorio-allantoic membranes after 32 days but not after 37 days; however, viable organisms were not found in the yolk sac stored for 29 days. The chorio-allantoic membranes which were stored 44 and 48 days also yielded viable toxoplasma. Mixed 10, 25, and 50 per cent Y.S.-C.A. suspensions were tested after storage at 4 C (Table 11). Ten per cent suspensions caused toxoplasma infection in mice after 8 days but not after 14 days of storage. The 25 per cent suspensions contained living parasites after 21 days but not after 28 or 29 days of storage. Four 50 per cent Y.S.-C.A. suspensions were tested. Viable organisms were obtained in these trials at 21, 27, 28, and 31 days, but not at 28, 40, 41, and 48 days, respectively. Data on the effect of the temperature of storage are given in table 12. Infected tissue which had been frozen rapidly by means of a dry ice-alcohol bath, and stored at -70 or -10 C produced no infection in mice at the first test. This is in agreement with previously observed failures to store infected mouse brain satisfactorily at dry ice temperatures. Whole yolk sac and chorio-allantoic

Table 10

Viability of Toxoplasma in Infected Embryos after Storage of Whole Membranes at 4 C

Egg Passage	Tissue Stored	Days of Storage													
		10	14	17	21	22	25	28	29	32	36	37	41	44	48
19	Yolk Sac		+		+			-			-				
	Chorio-allantoic Membrane		+		+			+			-				
5-48	Yolk Sac	+		+		+	+		-	+		-	-	-	-
	Chorio-allantoic Membrane	+		+		+	+		+	+		-	-	+	+

+ Recovery of toxoplasma by injection of mice.

- Failure to recover toxoplasma by injection of mice.

Table 11

Effect of Concentration on Survival of Toxoplasma in Y.S.-C.A. Suspensions at 4 C

Egg Passage	Suspension Stored	Days of Storage											
		7	8	12	14	21	27	28	29	31	40	41	48
18	10% Y.S.-C.A.		+		-								
	25% "		+		+	+		-					
	50% "		+		+	+		-					
10	25% Y.S.-C.A.			+					-				
7	50% Y.S.-C.A.	+			+	+				+			-
16	50% Y.S.-C.A.							+				-	
16	50% Y.S.-C.A.		+				+				-		

+ Recovery of toxoplasma by injection of mice.

- Failure to recover toxoplasma by injection of mice.

Table 12

Effect of Temperature on Survival of Toxoplasma in Infected Egg Tissues

Tissues from egg passage 10

Tissue Stored	Temperature of Storage	Days of Storage							
	°C	1	2	3	6	12	29	32	54
25% Y.S.-C.A.	-70		-						
	-10		-						
	4					+	-		
	25		+		+		-		
	37	+		+			-		
Whole Y.S.-C.A.	4					+		+	-
	25					+		-	
Whole Y.S.-C.A. with 5.0 ml of egg fluids	4					+		+	-
	25					+		-	

\+ Recovery of toxoplasma by injection of mice.

\- Failure to recover toxoplasma by injection of mice.

membranes also have been tested after dry ice storage and none have yielded viable organisms. Toxoplasma infection in mice has been produced by 25 per cent Y.S.-C.A. stored for 6 days at 25 C, room temperature, and for 3 days at 37 C. Whole yolk sac and chorio-allantoic membranes, with or without added egg fluids, yielded viable toxoplasma after 12 days at 25 C but not after 32 days; yet corresponding membranes stored at 4 C yielded viable organisms at 32 days. These periods of successful storage are, therefore, somewhat longer than the storage of infected mouse brain under Tyrode's solution as is commonly practiced. In our experience infected whole mouse brains stored at 4 C under Tyrode's solution have been infectious after 21 days but never for longer periods.

Serum neutralization tests to determine toxoplasma antibodies are generally performed according to the method of Sabin (9) which consists of intracutaneous injection into the rabbit's back of mixtures of undiluted serum and 1:10, 1:50, 1:500, and 1:5000 dilutions of toxoplasma infected mouse brain in Tyrode's solution. The degree of inhibition of skin lesions as compared to control lesions permits the degree of neutralization to be determined. Three neutralization tests were performed in eggs using death of chick embryos as the criterion for evaluating the neutralization index (Table 13). A freshly prepared 20 per cent mouse

brain suspension was obtained by shaking infected mouse brains with glass beads, and decimal dilutions were prepared from this. Equal quantities of the dilutions were then mixed with the undiluted serums to be tested and allowed to stand at room temperature for 20 minutes, after which 0.2 ml quantities of the mixture were injected into ten-day chick embryos either into the yolk sac or onto the chorio-allantoic membrane. Final dilutions of mouse brain thus injected were 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. In all three tests a normal rabbit serum was tested simultaneously with an immune rhesus monkey serum. The LD 50 titers were calculated for both the normal serum and the immune serum, and from these the neutralization index of the immune serum was determined. This index is the anti-log of the difference between the LD 50 titer of the normal serum and the LD 50 titer of the immune serum, both expressed as the logarithms of the infected mouse brain dilutions. Serum neutralizations performed by inoculation into the yolk sac gave neutralization indexes of greater than 300 with one serum and 32 for a second serum. Inoculation onto the chorio-allantoic membrane gave a neutralization index of 100. Both methods are therefore practicable, with the yolk sac method the easier to do; however neither is wholly practical since most serum specimens are not handled with the conditions of rigorous asepsis that are essential to prevent the interference by bacterial contamination of the

Table 13

Toxoplasma Serum Neutralization Test
in 10-Day Chick Embryos

0.2 ml of mixture of undiluted serum and mouse brain suspension per egg

Method of Inoculation of Eggs	Serum (Undiluted)	Dilution of Mouse Brain 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	LD_{50} Titer	Neut. Index
Y.S.	Normal Rabbit			1/1*	2/2	1/1		$>10^{-5.5}$	>300**
	Rhesus 2 (7 wk bleeding)		1/1	1/2	0/1			$10^{-3.0}$	
Y.S.	Normal Rabbit			3/3	2/3	0/1	0/2	$10^{-4.3}$	32
	Rhesus 2 (10 wk bleeding)		3/3	1/3	0/3			$10^{-2.8}$	
C.A.	Normal Rabbit		3/3	1/2	1/2	0/2		$10^{-3.5}$	100
	Rhesus 2 (10 wk bleeding)	1/1	0/2	0/2	0/1			$10^{-1.5}$	

* Numerator represents number of chick embryos dead from toxoplasma.
Denominator " " " " " inoculated (minus nonspecific deaths due to trauma or bacteria).

** Neutralization index by intracerebral injection of mice with the same serum-mouse brain mixtures was 15.

the results of serum neutralization tests in developing hen's eggs.

In view of the successful cultivation of the etiologic agent of granuloma inguinale in embryonic yolk by Anderson, DeMonbreun, and Goodpasture (17) two separate attempts were made to cultivate toxoplasma in the pooled embryonic fluids from seventeen-day incubated fertile eggs, with and without the addition of embryonic chick brain or heart. Both attempts were unsuccessful.

Passage thru eggs has not resulted in any obvious loss of pathogenicity for animals. After more than 13 passages egg tissues have caused the disease in mice, rats, two- to eleven-day chicks, rabbits, and a rhesus monkey.

With the adaptation of the R.H. strain of toxoplasma to growth in the embryonated egg, the various tissues of such eggs offered promise of being easily obtained antigens of high quality for use in the complement fixation reaction. In order to establish a base line from which to proceed, attempts were made to repeat the works of Nicolau and Ravelo (11) and of Warren and Sabin (12).

Nicolau and Ravelo prepared methanol extracts of desiccated, infected rabbit spleen, and reported specific fixation with antisera from toxoplasma-infected rabbits and other

animals. Methanol extracts were prepared in this laboratory by the same methods from an infected rabbit spleen and a normal rabbit spleen. The spleens were desiccated in vacuo for five days, and then extracted for 24 hours at 37 C using 25 ml of methanol per gram of dry spleen. The filtrates from these extracts constituted the antigen and the normal control extract. After the incubation at 37 C of 0.2 ml quantities of each of these extracts with 0.2 ml of 2 per cent sheep red blood cells and 0.6 ml of physiological saline the sheep red blood cells were partially hemolyzed. Therefore these antigens were considered unfit for use in the complement fixation reaction.

With sera from rhesus monkeys infected by intracutaneous and intra-abdominal injection, Warren and Sabin obtained specific fixation of complement with frozen and thawed saline extracts of infected rabbit brain. In order to show specific fixation it was nexessary for them to perform simultaneous tests, using a similar extract of normal rabbit brain. The complement fixing antibody appeared within 1 to 4 weeks after inoculation but, unlike the neutralizing antibody, disappeared again in most monkeys, sometimes even as early as 2 months thereafter. In view of the results reported by Warren and Sabin, similar experiments were attempted by the author.

Two rhesus monkeys were bled for normal serum, and were then infected with toxoplasma by the combined intracutaneous and intraperitoneal routes. Rhesus 1 received 10.0 ml of 10 per cent infected mouse brain intraperitoneally, and two simultaneous intracutaneous injections of 0.2 ml of the same suspension. Rhesus 2 was injected with the same quantities of a 10 per cent toxoplasma-infected yolk sac-chorio-allantoic membrane suspension. That both monkeys became infected was proven by elevated temperatures of five to seven days in duration, and 1.5 to 2.0 cm areas of induration at the sites of the intracutaneous injections. Weekly bleedings by cardiac puncture under ether anesthesia were performed during the first eight weeks after inoculation. Rhesus 1 was also bled at 12 weeks after inoculation, and rhesus 2 was bled at 10, 12, and 14 weeks after infection. Each blood specimen was allowed to clot, the serum was drawn off, placed in sterile lusteroid tubes, and frozen on the day of the bleeding. Such sera were kept frozen at dry ice temperatures until needed. This was the method of storage that was found most favorable by Sabin and Ruchman (10). Rabbit neutralization tests with the normal sera were negative. Equivocal neutralization was obtained with the one- and two-week sera of rhesus 1, and the one-week serum of rhesus 2. Positive neutralization was obtained with all sera obtained two weeks or more after inoculation of rhesus 2, and three

weeks after inoculation of rhesus 1.

Three sets of rabbit brain antigens were prepared in a manner similar to that described by Warren and Sabin (12). For the toxoplasma infected brains, rabbits weighing approximately 2 kilograms were injected intracerebrally with 0.4 or 0.5 ml of 10 per cent infected mouse brain. Upon death of the rabbit the brain was removed, ground with mortar and pestle, and a 10 per cent saline suspension prepared. After storage overnight at ordinary refrigerator temperature, about 4 C, the suspension was alternately frozen and thawed five times. A dry ice-alcohol bath was used to freeze, and running cold water was used to thaw the material. After centrifugation for 30 minutes at 3000 rpm in an angle centrifuge, the supernatant was removed and this constituted the antigen. Normal rabbit brains were processed similarly to serve as controls. These antigens and controls were stored at 4 C until used.

Procedure of the complement fixation test using rabbit brain antigens: Sheep red blood cells were washed four times in saline and a 2 per cent suspension prepared. Commercial hemolysin (Sharp and Dohme) was titrated with these red blood cells in an excess of complement, and the red blood cells were sensitized with an equal volume of the hemolysin dilution required to give 2 units of amboceptor per 0.2 ml; thus 0.4 ml

of sensitized erythrocytes contained 0.2 ml of 2 per cent cells and 0.2 ml of amboceptor (2 units). Hemolysin and complement titrations were incubated at 37 C for 30 minutes. Guinea pig complement was stored frozen, and was titrated immediately before use. For the test proper, the complement was diluted so that 0.2 ml contained 1 1/3 units. Immune sera were inactivated for 30 minutes at 56 C unless otherwise stipulated. To determine fixation, 0.2 ml of the immune serum dilution, 0.2 ml of complement (1 1/3 full units), and 0.2 ml of the undiluted rabbit brain antigen were incubated for 30 minutes at 37 C. Two-tenths ml of each serum dilution was incubated similarly with the normal rabbit brain suspension. Thereafter, 0.4 ml of sensitized red blood cells were added to each tube, and the degree of fixation was determined after an additional 30 minute incubation at 37 C. Complete fixation was designated as 4 and no fixation as 0, with various intermediate degrees between the two extremes designated as 3, 2, and 1 plus in the order of decreasing fixation of complement. Final readings were made after overnight refrigeration at 4 C. The usual anti-complementary and hemolytic controls were included in each test.

A typical protocol of a complement fixation test with monkey sera is given in table 14. Normal monkey serum fixed complement in low dilutions, 1:8 or less, in the presence of both the toxoplasma rabbit brain antigen and the normal rabbit

Table 14

Complement Fixation with Monkey Sera Employing Rabbit Brain Antigens

Rhesus	Weeks after Inoculation	Antigen or Control	Serum Dilution							
			Undil.	1:2	1:4	1:8	1:16	1:32	1:64	a.c. Undil.
1	Normal	Tox. Rabbit Brain 1A	1	1	0	0	0	0	0	0
		Normal " " "	0	1	0	0	0	0	0	
	1	Tox. " " "	3	3	3	3	3	1	0	0
		Normal " " "	3	3	3	3	3	1	0	
	2	Tox. " " "	3	3	3	3	1	0	0	0
		Normal " " "	3	3	3	3	1	0	0	
	3	Tox. " " "	4	4	4	4	4	2	0	0
		Normal " " "	4	4	4	4	2	1	0	
	4	Tox. " " "	4	4	4	4	4	4	0	0
		Normal " " "	4	4	4	4	4	2	0	
	6	Tox. " " "		4	4	4	4	1	0	0
		Normal " " "		4	4	4	3	0	0	
										a.c. 1:2
2	Normal	Tox. Rabbit Brain 1A		3	2	1	0	0		0
		Normal " " "		3	2	1	0	0		
	2	Tox. " " "		3	3	3	1	0	0	0
		Normal " " "		3	3	3	1	0	0	
	4	Tox. " " "		3	3	3	1	0	0	0
		Normal " " "		3	3	3	1	0	0	
	5	Tox. " " "		3	3	3	1	0	0	0
		Normal " " "		3	3	3	1	0	0	

control. This nonspecific fixation was higher in rhesus 2. Sera obtained 1 to 6 weeks after inoculation of the monkeys with toxoplasma, either with infected chick embryo material or mouse brain, fixed complement in higher dilutions, 1:16 and 1:32. The differences between the degrees of fixation with the toxoplasma infected brain antigen and the normal control brain preparation were in all cases less than 2-fold, and in most cases identical degrees of fixation were obtained with the toxoplasma and normal brain antigens. Similar data were obtained with rabbit brain antigens 2A and 3A and their corresponding controls. Since these antigens were prepared from different rabbit brains, the possibility that individual variation among rabbits had effected the results was reduced. Rabbit brain antigen 3A and its control were further processed by the method utilized by Sabin for the preparation of a complement fixing antigen for Japanese B encephalitis (18). This consisted of repeated freezing and thawings, each followed by angle centrifugation at 3500 rpm. Toxoplasma rabbit brains and controls treated in this manner did not alter the basic pattern of fixation. The only notable change was that all sera fixed complement 1 tube lower (2-fold less) than the original antigen or control. In view of the large amount of nonspecific fixation it would be hazardous to grant significance to the difference between the 4- and 2-plus fixations with

rhesus 1 sera at the third and fourth week bleedings in dilutions of 1:16 and 1:32, respectively, yet these differences were in the direction of specific fixation.

Table 15 gives a direct comparison of rabbit brain antigens 1A and 2A and normal rabbit brain 2A with the same sera. The difference in fixation is as great between antigens as between antigen 1A and control preparation 2A.

In view of the high lipoid content of brain material the effect of ether extraction upon toxoplasma rabbit brain antigens was investigated. Toxoplasma rabbit brain antigen 1A was extracted with an equal volume of ethyl ether at room temperature for 72 hours, the aqueous material was separated and the residual ether was removed by slight vacuum. Normal rabbit brain 1A was treated identically. No fixation of complement was obtained with either the toxoplasma or normal material in the lowest serum dilutions tested (Table 15); thus ether extraction removed all the nonspecific, and possibly the specific, complement fixing properties of rabbit brain antigens.

In many complement fixation tests it is essential to titrate complement in the presence of the antigen. Titration of 1:10 complement in the presence of toxoplasma rabbit brain antigen 2A yielded the same titer as the titration in the

Table 15

Comparison of Rabbit Brain Antigens
(Rhesus 1 immune sera)

Weeks after Inoculation	Antigen or Control	Serum Dilutions 1:5	1:10	1:20	1:30	1:40	1:50	1:60	a.c. 1:5
Normal	Tox. Rabbit Brain 1A	2	1	0	0				0
	" " " 2A	2	2	0	0				
	Normal " " 2A	3	2	1	0				
	Ether Ext. Tox. Rabbit Brain 1A	0	0	0	0				
	" " Normal " " 1A	0	0	0	0				
4	Tox. Rabbit Brain 1A	4	4	4	4	2	0	0	0
	" " " 2A	4	4	4	4	3	0	0	
	Normal " " 2A	4	4	4	4	1	0	0	
	Ether Ext. Tox. Rabbit Brain 1A	0	0	0	0	0	0	0	
	" " Normal " " 1A	0	0	0	0	0	0	0	
8	Tox. Rabbit Brain 1A	4	4	4	2	1	0	0	0
	" " " 2A	4	4	4	3	0	0	0	
	Normal " " 2A	4	4	4	1	0	0	0	
	Ether Ext. Tox. Rabbit Brain 1A	0	0	0	0	0	0	0	
	" " Normal " " 1A	0	0	0	0	0	0	0	

presence of saline only (Table 16). This was also true for normal rabbit brain 2A. In all three cases one full unit of complement was 0.04 ml; however if both toxoplasma rabbit brain 2A and undiluted rabbit serum were present, it required 0.09 ml of 1:10 complement for 1 full unit. The same was true when complement was titrated in the presence of normal rabbit materials. Experiment 2 of table 16 showed that this anti-complementary effect of the rabbit brain-normal serum combination was even more pronounced if the complement dilutions were refrigerated overnight at 4 C before the addition of the sensitized cells.

When serum from the six-week bleeding of rhesus 1 was tested for fixation, using two complement concentrations, two full units of complement yielded less fixation with both the toxoplasma and normal antigens than was obtained with the more sensitive test employing 1 1/3 full units; yet the difference between the fixation with toxoplasma antigen and the normal brain material (specific fixation) was identical with both 1 1/3 and 2 units of complement.

Casals and Palacios (19) increased the specificity of their complement fixation tests in viral diseases by heating the immune sera to temperatures higher than the usual 56 C used for inactivation. By inactivation of a 1:4 dilution of rhesus serum at 65 C for 30 minutes (Table 17) all of the non-

Table 16

Titration of Complement in Presence
of Rabbit Brain Antigens and Normal Rabbit Serum

Reagents Added to Titration	1:10 Complement Equal to 1 Full Unit		
	Experiment 1	Experiment 2	
	ml	37 C, 30 Min ml	0.N. 4 C ml
Saline	.04	.05	.04
" , 0.2 ml Tox. Rabbit Brain 2A	.04	.05	.04
" , 0.2 ml Tox. Rabbit Brain 2A, 0.2 ml Undil Normal Rabbit Serum	.09	.08	.10
" , 0.2 ml Normal Rabbit Brain 2A	.04	.05	.04
" , 0.2 ml Normal Rabbit Brain 2A, 0.2 ml Undil Normal Rabbit Serum	.09	.08	.10
" , 0.2 ml Supernatant* Rabbit Brain 2A	.04		
" , 0.2 ml Supernatant* Rabbit Brain 2A, 0.2 ml Undil Normal Rabbit Serum	.08		
" , 0.2 ml Supernatant* Normal Rabbit Brain 2A	.04		
" , 0.2 ml Supernatant* Normal Rabbit Brain 2A, 0.2 ml Undil Normal Rabbit Serum	.08		

* This is the designation applied to the antigen prepared according to Sabin's method for Japanese B encephalitis (18).

specific fixation was eliminated for normal serum in the presence of toxoplasma and control rabbit brain. With monkey serum obtained six weeks after inoculation with toxoplasma nonspecific fixation was present after inactivation at 65 C, but in lower titer and the degree of specific fixation, if there was any, was slightly increased; yet this specific fixation was still less than 2-fold. However, when an entire test was set up using only sera inactivated at 65 C for 30 minutes, very unusual results were obtained (Table 18). No fixation, specific or nonspecific, occurred in lower dilutions, yet 1- and 2-plus fixation occurred in the higher dilutions, and in all probability these results represented nonspecific fixation. No explanation is apparent for these results, the only difference between the tests quoted in tables 17 and 18 being in the antigens used.

In an attempt to explain some of the nonspecific reactions obtained with monkey sera, the sera were titrated for their ability to hemolyze sheep red blood cells (Table 19). These titrations were performed by mixing 0.2 ml of inactivated serum dilutions with 0.2 ml of 1:10 complement, an excess, and 0.2 ml of 2 per cent washed sheep erythrocytes; bringing the total volume to 1.0 ml with saline. After incubation at 37 C for 1 hour the results were read: 0 for complete hemolysis and 4 for no hemolysis. All sera tested, including

Table 17

Complement Fixation with Rhesus 1 Sera and Rabbit Brain Antigens

Variation of Units of Complement and Temperature of Inactivation of Sera

Weeks after Inoculation	Antigen or Control	Serum Inactivation °C	Full Units of Complement	Serum Dilutions 1:4	1:8	1:16	1:32	1:64	a.c. 1:4
Normal	Tox. Rabbit Brain 2A	65	1 1/3	0	0	0	0		0
	Normal " " "	"	" "	0	0	0	0		
	Tox. " " "	"	2	0	0	0	0		
	Normal " " "	"	"	0	0	0	0		
6	Tox. Rabbit Brain 2A	56	1 1/3	4	4	4	4	1	0
	Normal " " "	"	" "	4	4	4	3	0	
	Tox. " " "	"	2	4	4	4	1	0	
	Normal " " "	"	"	4	4	3	0	0	
	Tox. " " "	65	1 1/3	4	4	1	0	0	0
	Normal " " "	"	" "	4	2	0	0	0	
	Tox. " " "	"	2	4	2	0	0	0	
	Normal " " "	"	"	4	0	0	0	0	

Table 18

Complement Fixation with Monkey Sera
after Inactivation at 65 C for 30 Minutes

Rabbit Brain Antigens

Rhesus	Weeks after Inoculation	Antigen or Control	Serum Dilution 1:4	1:8	1:16	1:32	1:64	a.c. 1:4
1	Normal	Tox. Rabbit Brain 3A	0	0	1	2	2	0
		Normal " " "	0	0	0	0	-	
	2	Tox. " " "	0	0	0	1	1	0
		Normal " " "	0	0	0	0	0	
	4	Tox. " " "	0	0	1	0	0	0
		Normal " " "	0	0	0	0	0	
	7	Tox. " " "	0	0	0	2	2	0
		Normal " " "	1	0	1	2	2	
2	Normal	Tox. Rabbit Brain 3A	0	0	0	2	2	0
		Normal " " "	0	0	0	1	1	
	2	Tox. " " "	0	0	0	2	2	0
		Normal " " "	0	0	0	1	2	
	4	Tox. " " "	0	0	1	2	1	0
		Normal " " "	0	0	1	1	2	
	8	Tox. " " "	0	0	0	2	2	0
		Normal " " "	0	2	0	2	2	
	12	Tox. " " "	0	1	2	2	2	0
		Normal " " "	0	1	2	2	-	

Table 19

Titration of Monkey Sera for
Sheep Red Blood Cell Hemolysins

Rhesus	Weeks after Inoculation	Serum Inactivation °C	Serum Dilution 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
1	Normal	56	0*	0	0	3	4	4	4	4
	2	"	0	0	0	0	0	0	1	4
	4	"	0	0	0	0	0	4	4	4
	6	"	0	0	0	0	1	4	4	4
		65		4	4	4	4	4	4	4
	8	56	0	0	0	1	2	4	4	4
2	Normal	56	0	0	0	1	2	3	4	
		"	0	0	0	0	2	2	2	
	2	65	0	4	4	4	4	4	4	
	4	56	0	0	0	0	1	2	3	
	8	"	0	0	0	0	2	3	4	
	12	"	0	0	0	1	2	3	4	

* 0 indicates complete hemolysis of sheep red blood cells.

4 " no " " " " " "

normal monkey sera, were found to be hemolytic; however sera obtained 2 to 4 weeks after inoculation with toxoplasma contained considerably more hemolysin than did normal monkey sera or sera obtained 8 to 12 weeks after toxoplasma inoculation. Inactivation of sera at 65 C for 30 minutes completely removed this hemolysin in all but the 1:2 dilution. Similarly, adsorption of the inactivated sera overnight at 4 C with 3 volumes of 10 per cent washed sheep red blood cells removed this hemolytic property. When a complete complement fixation test was performed with sera adsorbed in this manner, unusual results were obtained (Table 20), similar to those observed with sera inactivated at 65 C.

If white rats are injected intracerebrally with toxoplasma they die, and the brains of such animals are quite heavily parasitized. Four rats of approximately 100 gm each were injected intracerebrally with 0.04 ml of 10 per cent infected mouse brain. On the fourth day these rats were sick and showed signs of central nervous system involvement, so they were sacrificed. The brains were removed aseptically and a complement fixing antigen was prepared from them. This was done by grinding, diluting with saline to 10 per cent, freezing and thawing five times, and centrifuging for 30 minutes at 3500 rpm in an angle centrifuge. The supernatant after centrifugation was the

Table 20

Complement Fixation with Sera of Rhesus 2
Which Had Been Absorbed with Sheep Red Blood Cells

Rabbit Brain Antigens

Weeks after Inoculation	Antigen or Control	Serum Dilution 1:4	1:8	1:16	1:32	1:64	a.c. 1:4
Normal	Tox. Rabbit Brain 3A	0	0	0	0		0
	Normal " " "	0	0	0	0		
2	Tox. " " "	0	1	1	1	1	0
	Normal " " "	0	1	0	1	1	
2*	Tox. " " "		2	2	2	2	0
	Normal " " "		2	2	2	2	
4	Tox. " " "	0	2	1	2	2	0
	Normal " " "	0	2	2	2	2	
8	Tox. " " "	0	2	2	2	2	0
	Normal " " "	0	1	2	2	2	
12	Tox. " " "	0	2	2	2	2	0
	Normal " " "	0	1	2	1	2	

* This 2-week serum had not been absorbed but had merely been inactivated.

antigen. Two brains from normal rats were processed similarly to serve as the control. Table 21 lists the results obtained with these rat brain preparations when allowed to fix complement in the presence of monkey sera. No nonspecific fixation was obtained with normal monkey serum. Nonspecific fixation was obtained with the immune sera from rhesus 1 but not with the immune sera from rhesus 2. The explanation for this difference might be that rhesus 1 had originally received mouse brain material whereas rhesus 2 had received a chick embryo suspension; therefore, the rat brain antigen, being more closely related to the mouse brain, produced more nonspecific fixation in rhesus 1. Again the degree of specific fixation was questionable, and less than 2-fold.

One frozen and thawed antigen was prepared from infected chorio-allantoic membranes but was stored as a crude 10 per cent suspension in the refrigerator at 4 C for 70 days. After this period of storage the antigen and its normal chorio-allantoic control were centrifuged and the supernatant used in a complement fixation test with the four-week bleeding of rhesus 1. Nonspecific fixation with both the toxoplasma and normal chorio-allantoic membranes preparations occurred in the 1:4 and 1:8 serum dilutions. As this long period of storage might have permitted degradation of the specific antigen, or increase in the nonspecific reaction of the

Table 21

Complement Fixation with Monkey Sera
Employing Rat Brain Antigens

Rhesus	Weeks after Inoculation	Antigen or Control	Serum Dilution 1:4	1:8	1:16	1:32	1:64	1:128	a.c. (1:4)
1	Normal	Tox. Rat Brain 1	0	0	0	0			0
		Normal " " "	0	0	0	0			
	2	Tox. " " "	4	4	3	0	0	0	0
		Normal " " "	4	4	3	1	0	0	
	4	Tox. " " "	4	4	2	2	2	0	0
		Normal " " "	3	2	2	2	1	0	
	7	Tox. " " "	4	2	1	1	0	0	0
		Normal " " "	3	0 (?)	1	1	0	0	
2	Normal	Tox. Rat Brain 1	0	0	0	0			0
		Normal " " "	0	0	0	0			
	2	Tox. " " "	2	0	0	-	-	0	0
		Normal " " "	0	0	0	-	-	0	
	4	Tox. " " "	0	0	0	0	0	0	0
		Normal " " "	0	0	0	0	0	0	
	12	Tox. " " "	0	0	0	0	0	0	0
		Normal " " "	0	0	0	0	0	0	

antigen, this was not deemed good evidence on which to discard the possibility of use of chick embryo tissues as complement fixing antigens.

Preparation of yolk sac-chorio-allantoic membrane antigen: The pooled yolk sacs and chorio-allantoic membranes of five chick embryos dead five days after inoculation of ten-day embryos with egg passage 21 had been frozen immediately after harvest, and stored in the dry ice chest. Similarly, the pooled yolk sacs and chorio-allantoic membranes from three 15-day normal chick embryos had been frozen immediately after harvest and stored in the dry ice chest. These membranes were removed from the dry ice chest the same day, thawed, and each group of membranes was blended in a Waring blendor for three minutes with the required saline to give a 10 per cent Y.S.-C.A. suspension. The resulting suspensions were centrifuged in an angle centrifuge at 8000 rpm for 30 minutes. Three phases resulted from centrifugation, a heavy sediment, a middle aqueous phase, and a light yolk top layer. The aqueous phases were removed and the few large yolk fragments which passed into these phases were removed by filtration through two layers of sterile gauze. The resulting suspensions constituted toxoplasma Y.S.-C.A. antigen 1 and normal Y.S.-C.A. 1.

Complement fixation tests were set up with these two

preparations as follows: 0.2 ml of serum dilutions, 0.2 ml of complement containing 2 exact units, and 0.2 ml of antigen or control were incubated in the 37 C water bath for 30 minutes, 0.4 ml of sensitized red blood cells added and the test read after an additional 30 minutes at 37 C. Normal monkey sera did not fix complement with either the toxoplasma or normal material (Table 22). Rhesus sera obtained two weeks after inoculation fixed complement with both antigen and normal Y.S.-C.A. The antigen yielded the higher titer but the specific fixation amounted to less than two-fold. However, sera obtained from both monkeys at 5- and 8-week intervals after inoculation gave only specific fixation. Rhesus 1 fixed complement at 5 and 8 weeks to a dilution of 1:16. Rhesus 2 at 5 weeks also had a titer of 1:16; however, at 8 weeks this titer had dropped to 1:8, and no fixation was obtained in a 1:4 dilution of the 14-week serum. Specific complement fixation, therefore, was obtained with antigens prepared from infected chick embryos.

An antigen was prepared from three infected chorio-allantoic membranes stored as whole membranes at 4 C for six months. The membranes were blended for 3 minutes in the Waring blendor with saline and merthiolate to give final concentrations of 10 per cent C.A. and 1-10,000 preservative. This was then centrifuged 30 minutes at 8000 rpm. The super-

Table 22

Complement Fixation with Monkey Sera
Employing Yolk Sac-Chorioallantoic Membrane Antigen 1

Rhesus	Weeks after Inoculation	Antigen or Control	Serum Dilution 1:4	1:8	1:16	1:32	1:64	a.c. 1:4
1	Normal	Tox. Y.S.-C.A. 2	0	0	0	0	0	0
		Normal "	0	0	0	0	0	
	2	Tox. "	4	4	4	1	1	1
		Normal "	4	4	2	0	0	
	5	Tox. "	4	4	3	0	0	0
		Normal "	0	0	0	0	0	
	8	Tox. "	4	4	2	1	0	0
		Normal "	0	0	0	0	0	
2	Normal	Tox. Y.S.-C.A. 2	0	0	0	0	0	0
		Normal "	0	0	0	0	0	
	2	Tox. "	4	4	4	2	0	0
		Normal "	4	4	2	0	0	
	5	Tox. "	4	4	3	0	0	0
		Normal "	0	0	0	0	0	
	8	Tox. "	4	2	0	0	0	0
		Normal "	0	0	0	0	0	
	14	Tox. "	0	0	0	0	0	0
		Normal "	0	0	0	0	0	

natant fluid constituted toxoplasma C.A. antigen 2. A control was prepared simultaneously, using whole normal chorio-allantoic membranes of the same age stored at 4 C for eight months.

In order to test the effect of merthiolate and ether extraction, a crude toxoplasma-infected Y.S.-C.A. suspension and the corresponding control were prepared by blending. These were divided and processed in three ways. One fraction was diluted to 10 per cent Y.S.-C.A. concentration and centrifuged. To the second fraction 1-1,000 merthiolate and saline were added to give final concentrations of 10 per cent Y.S.-C.A. and 1-10,000 preservative and this was centrifuged. The third fraction was handled just as was the second, but was further processed by extraction with ether. An equal volume of C.P. ethyl ether was shaken with the toxoplasma Y.S.-C.A. centrifugate and allowed to separate overnight at 4 C. After separation of the ether phase, the residual ether in the aqueous phase was removed by placing the suspension in a 40 C water bath for 10 minutes. The aqueous phase then constituted the complement fixing substance.

Comparative fixation tests were performed with the above three Y.S.-C.A. preparations, the chorio-allantoic reagents described previously, and the rabbit brain suspensions (Table 23). Specific fixation to the same degree was obtained with

the toxoplasma Y.S.-C.A., with or without added merthiolate, and no nonspecific fixation was observed. Ether extraction completely removed the ability to fix complement. A specific fixation of an 8-fold degree was demonstrated with the chorio-allantoic antigen that was prepared from whole membranes which had been stored at 4 C for six months, regardless of the non-specific fixation in the 1:4 and 1:8 serum dilutions. With the same serum the rabbit brain material again gave nonspecific fixation to a dilution of 1:16, and specific fixation of less than 2-fold. Consequently all further efforts were directed toward enhancing both the specificity and the sensitivity of the antigens prepared from tissues of toxoplasma-infected developing chick embryos. Since the highest titers of infectivity had been found to occur in the chorio-allantoic membrane, and since this membrane was relatively free of the troublesome yolk constituents, all new antigens were prepared from chorio-allantoic membranes.

Preparation of complement fixing antigens from chorio-allantoic membranes: Chorio-allantoic membranes were harvested immediately after embryo deaths and were either immediately processed to antigen or stored in the frozen state until time permitted processing. All membranes were from embryos which died either five or six days after inoculation of ten-day fertile eggs with the R.H. strain of toxoplasma. These membranes bore

Table 23

Comparison of Chick Embryo Antigens
with Rabbit Brain Antigens

(Rhesus 1, 5-week serum, inactivated 20 minutes at 56 C)

Antigen or Control	Serum Dilution					
	1:4	1:8	1:16	1:32	1:64	a.c 1:4
Tox. Y.S.-C.A. 2	3	2	1	0	0	0
Normal "	0	0	0	0	0	
Tox. Y.S.-C.A. 2 with 1:10,000 Merthiolate	3	2	1	0	0	0
Normal " " " "	0	0	0	0	0	
Ether Extracted Tox. Y.S.-C.A. with 1:10,000 Merthiolate	0	0	0	0	0	0
" " Normal " " " "	0	0	0	0	0	
Tox. C.A. 2	4	4	4	3	2	0
Normal "	4	4	0	0	0	
Tox. Rabbit Brain 2A	4	4	4	2	0	0
Normal " " "	4	4	3	0	0	

numerous yellow-white plaques, and either titrated or would have titrated to $10^{-4.0}$ or $10^{-5.0}$ in mice. Membranes from normal 15-day fertile eggs were used as controls for toxoplasma membranes harvested 5 days after injection of ten-day fertile eggs. Similarly, membranes from normal 16-day fertile eggs were used as controls for the toxoplasma membranes harvested 6 days after injection. Two methods of processing were utilized. The method described by Warren and Russ (20) was used but with some variations. The membranes were ground in a mortar with sterile alundum, and a suspension was prepared in physiological saline with sufficient merthiolate to give a final concentration of 1-10,000 preservative and 20 per cent chorio-allantoic membrane. This suspension was then frozen and thawed three times and was centrifuged for 30 minutes at 5000 rpm in an angle machine. The supernatant constituted the antigen. The second, and preferable, method of antigen preparation differed from the above in that membranes were macerated by blending in a Waring blendor for five minutes in enough physiological saline to give 33.3 per cent suspension. Saline and merthiolate were added to give final concentrations of 20.0 per cent chorio-allantoic membrane and 1-10,000 preservative, and the suspension was frozen and thawed and centrifuged as in the first method. Antigens and controls were stored in the frozen state

at dry ice temperatures. At the time of use these antigens were further diluted with saline, generally to a 10 per cent concentration. Such antigens have never been found to be anticomplementary.

Modification of complement fixation procedure: In order to increase the sensitivity of the fixation test, and to make the procedure similar to that performed in most serological laboratories, the following changes were adopted for the tests hereafter reported. Two exact units of complement in 0.2 ml were mixed with 0.2 ml of serum dilutions and 0.2 ml of antigen, or control, and the mixtures were allowed to stand overnight at 4 C. Thereafter 0.4 ml of sensitized cells were added, and the results were read after incubation for 30 minutes at 37 C in a water bath. The sensitized cell suspensions were mixtures of equal parts of 3.0 per cent washed sheep red blood cells and a hemolysin dilution which contained two units of amboceptor per 0.2 ml. This is essentially the procedure for the standard Kolmer complement fixation reaction.

The toxoplasma chorio-allantoic membrane antigen 3 which was prepared by grinding was tested against rhesus 1 sera and immune sera obtained from white rats at two and at five weeks after intraperitoneal injection of 10 per cent toxoplasma-infected mouse brain (Table 24). Ruchman (21)

has shown that adult white rats survive intraperitoneal injection with living toxoplasma, and that their sera contain neutralizing antibodies as early as one week after injection. Such antibodies may be demonstrated for months thereafter. Normal serum from rhesus 1 did not fix complement with toxoplasma C.A. 3 but the 5-week serum gave specific fixation of 4- to 8-fold, and nonspecific fixation in the 1:4 and 1:8 dilutions. The sera from the white rats fixed complement even more strongly than did the rhesus serum. Rat serum obtained two weeks after toxoplasma injection fixed complement specifically 8-fold but did give nonspecific reactions in the 1:4 and 1:8 dilutions. However, serum obtained from the same rats five weeks after injection gave no nonspecific reaction and fixed complement specifically to a dilution of 1:64. This experiment demonstrated two facts: (a) the chorioallantoic membrane antigens were wholly suitable for comprehensive complement fixation studies provided that a normal control preparation was titrated simultaneously; (b) toxoplasma-infected white rats should be thoroughly investigated with regard to the ability of their sera to fix complement.

For purposes of standardizing all future toxoplasma complement fixation tests, a standard immune serum was prepared. A group of white rats was infected with the R.H. strain of toxoplasma by the peripheral route. Each rat

Table 24

Complement Fixation with Toxoplasma C.A. Antigen 3

Rhesus 1 and White Rat Sera

Serum	Weeks after Injection	Antigen or Control	Serum Dilution 1:4	1:8	1:16	1:32	1:64	1:128	a.c. 1:4
Rhesus 1	Normal	Tox. C.A. 3	0	0	0	0			0
		Normal "	0	0	0	0			
	5	Tox. "	4	4	4	2	0	0	0
		Normal "	3	1	0	0	0	0	
White Rats 1, 2, and 3 (Pooled Serum)	2	Tox. "	4	4	4	4	3	0	0
		Normal "	4	2	0	0	0	0	
	5	Tox. "	4	4	4	4	3	0	0
		Normal "	0	0	0	0	0	0	

received intraperitoneally 1.0 ml of a 10 per cent infected mouse brain suspension. Four weeks later each of 13 rats was bled from the heart for 3 to 4 ml of blood. After separation the sera were pooled, and all but a small quantity was frozen and stored in the dry ice chest. The remainder of the serum pool was stored at 4 C. Table 25 demonstrates that this standard serum titered the same, after 5 and 45 days of storage at dry ice temperatures, as did the fresh serum. Forty days of storage in an ordinary refrigerator at 4 C eliminated a 2-plus fixation in the 1:128 dilution, yet the fixation in the 1:32 and 1:64 dilutions was identical to that of fresh or frozen serum. The frozen standard serum was included in all tests hereinafter described with the exception of the tests on the sera from white rats 1, 2, 3, and 4. These will be discussed later. In all tests the standard serum gave 4-plus fixation up to and including the 1:32 dilution and a 3-plus fixation in the 1:64 dilution. Therefore, titers of all sera discussed in the remainder of this paper may be compared directly with each other with the exception of the titers quoted in tables 29 and 30.

This standard serum permitted standardization of the various antigens. Two units of antigen were arbitrarily set as the highest dilution of antigen which in 0.2 ml amounts would give a 4-plus fixation with 0.2 ml of a 1:32 dilution

Table 25

Storage of Standard Rat Serum

(Inactivated for 20 minutes at 56 C before each test)

Method of Storage	Length of Storage	Dilutions of Standard Rat Serum					
		1:8	1:16	1:32	1:64	1:128	a.c.
	days						
Fresh Serum (4 C)	1	4	4	4	3	2	0
Frozen with Dry Ice	5	4	4	4	3	2	0
" " " "	45		4	4	3	2	0
4 C (Refrigerator)	40	4	4	4	3	0	0

and a 3-plus fixation with 0.2 ml of a 1:64 dilution of the standard rat serum. Two antigens were used throughout the remainder of these experiments. With each of these, two units were contained in 0.2 ml of a 1:2 dilution of the antigen (Table 26), that is,, 0.2 ml of a 10 per cent antigen contained 2 units. These antigens (Tox. C.A. 3 and Tox. C.A. 4) were prepared according to the two methods described previously, by blending and by grinding; therefore, these two methods are equally good for the preparation of toxoplasma antigens. Furthermore, either antigen could be diluted 1:4 without appreciably lowering the degree of fixation. However, a 1:8 dilution of antigen resulted in considerable loss of potency. Dilutions of antigen to 1:16 and 1:32 removed completely their ability to fix complement with the lowest dilution of standard serum employed,, 1:16.

The comparative effects of storage at 4 C and in dry ice were tested on one antigen (Table 27). This antigen fixed complement in the presence of the standard serum equally well after 24 days of storage both at dry-ice temperature and at 4 C, and after 58 days of storage at 4 C. No antigen has yet lost potency during storage in dry ice,, but no tests on periods of storage longer than two months have been made.

The titration of complement in the presence of either

Table 26

Comparative Titrations of Chorio-allantoic Membrane Antigens Prepared by Grinding and Blending

Antigen	Antigen Dilution	Dilutions of Standard Rat Serum				
		1:16	1:32	1:64	1:128	a.c. 1:16
Normal C.A. 3 (Ground)	1:2	0	0	0	0	0
Tox. C.A. 3 (Ground with alundum)	Undil	4	4	3	1	
	1:2	4	4	3	1	
	1:4	4	4	2	1	
	1:8	4	2	0	0	
	1:16	0	0	0	0	
	1:32	0	0	0	0	
Tox. C.A. 4 (Blended for 5 minutes)	Undil	4	4	3	1	
	1:2	4	4	3	0	
	1:4	4	4	2	0	
	1:8	4	3	0	0	
	1:16	0	0	0	0	
	1:32	0	0	0	0	

Table 27

Storage of Toxoplasma C.A. Antigen 5

Antigen diluted to 10% C.A. for test

Method of Storage	Length of Storage	Dilutions of Standard Rat Serum			
		1:16	1:32	1:64	1:128
	days				
20% Antigen Kept Frozen with Dry Ice	24	4	4	0	0
20% Antigen at 4 C	"	4	4	0	0
20% Antigen at 4 C	58	4	4	0	0

toxoplasma chorio-allantoic membrane antigens and normal control preparations has always resulted in the same complement titer as was obtained by titration only in the presence of saline (Table 28).

Complement fixation has been performed with sera from rhesus monkeys, white rats, wild rats, pigeons, guinea pigs, chickens, rabbits,, and human beings using the standardized antigens and the standard immune rat serum to control variation from test to test.

Rhesus Monkey Sera: Sera from the same two immune rhesus monkeys that were studied in the preliminary experiments were tested with chorio-allantoic membrane antigen 3 and its control. In table 32 the titers are plotted for both the toxoplasma antigen and the control. This was necessary in order to demonstrate the specific fixation. Normal monkey sera fixed complement nonspecifically. After inoculation with toxoplasma, rhesus 1 and 2 both exhibited specific fixation and nonspecific fixation; however sera from rhesus 2 consistantly gave more nonspecific fixation and less specific fixation than sera from rhesus 1. This might be explained on the grounds that rhesus 2 was infected with chick embryo tissue, and that subsequently its serum was tested with almost homologous tissue. Nevertheless the high degree of nonspecific fixation with normal serum from rhesus

Table 28

Titration of Complement in Presence
of Chorio-allantoic Antigens

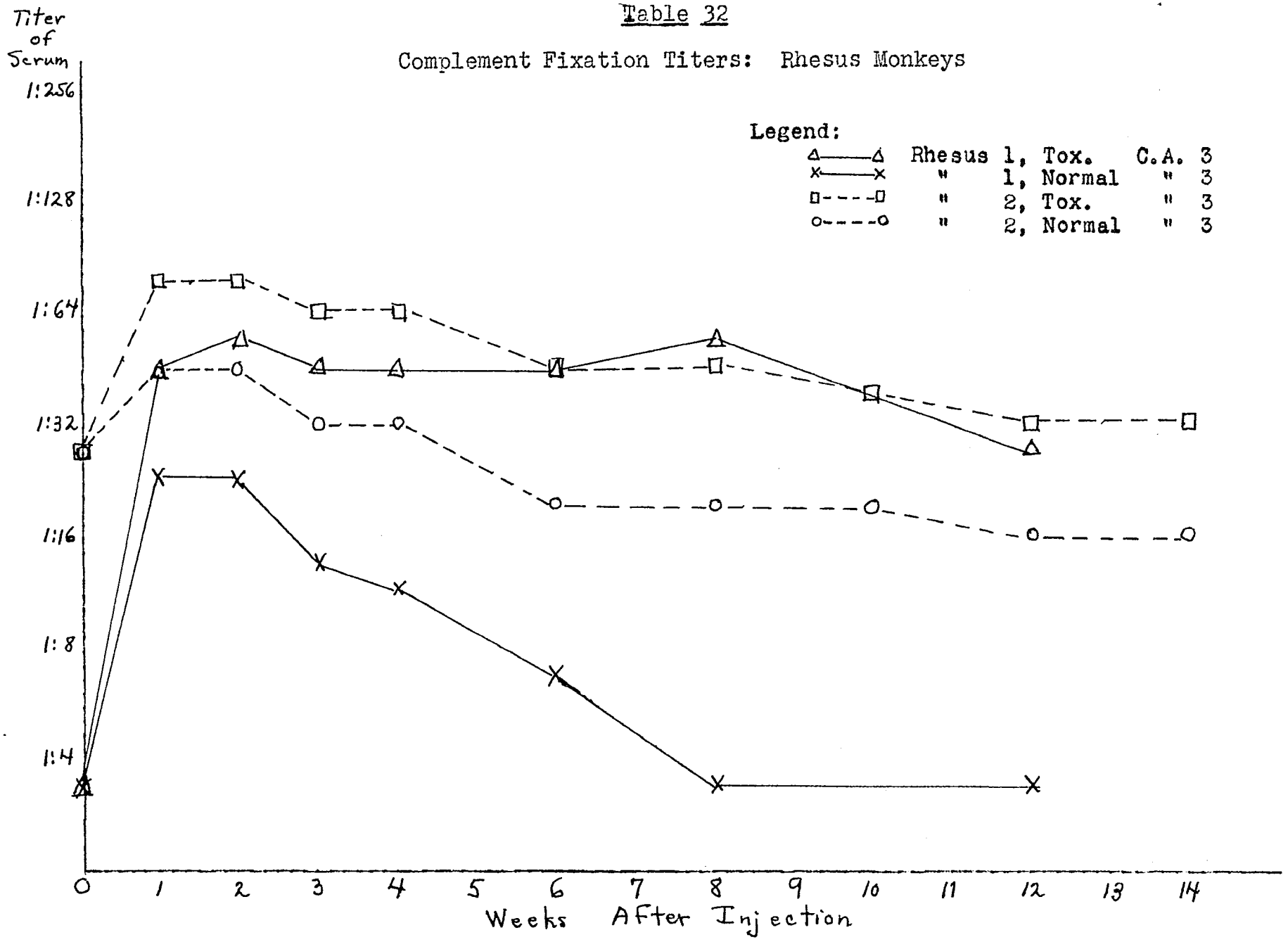
Reagents Present in Titration Before Addition of Sensitized R.B.C.*	1:30 Complement ml 0.06	0.09	0.12	0.15	0.18	0.21	0.24
1:30 Complement Saline	2	Trace	0**	0	0	0	0
1:30 Complement 0.2 ml Tox. C.A. 3 (per tube) Saline	2	"	0	0	0	0	0
1:30 Complement 0.2 ml Normal C.A. 3 (per tube) Saline	2	"	0	0	0	0	0

** 0 means complete hemolysis of 3% r.b.c.

* Reagents added in such a manner that all tubes contained a total of 0.6 ml before addition of sensitized sheep cells.

Table 32

Complement Fixation Titers: Rhesus Monkeys



2 could not be explained by this hypothesis. Nonspecific fixation of rhesus 1 sera was most pronounced between the first and sixth weeks but this had become insignificant by the eighth week. With the disappearance of the nonspecific fixation the degree of specific fixation became more obvious, increasing from 2-fold at 1 week to 16-fold at eight weeks. Rhesus 2 evidenced but slightly more than a 2-fold specific fixation. Both monkeys at the time of sacrifice, at 12 and at 14 weeks, possessed complement binding antibodies. Because of the large degree of nonspecific fixation in these monkeys, it was more difficult to demonstrate complement fixing antibodies in their sera than in the sera of white rats.

White Rat Sera: Adult white rats were bled for normal serum and were then inoculated intraperitoneally with a 10 per cent infected mouse brain. Rats 1, 2, 3, and 4 received 1.0 ml each, rats 5 and 6 received 2.0 ml each, and rats 7 and 8 were injected with 3.0 ml each. At various time intervals thereafter these rats were bled by cardiac puncture. All sera were separated on the day of bleeding and were kept frozen until tested.

Table 29 represents a typical protocol of complement fixation tests on sera from rats 1 and 2. Rat 1 exhibited no nonspecific fixation with the control chorio-allantoic preparation, and gave specific fixation in 1:64 and 1:128

Table 29

Complement Fixation with Sera from
White Rats Injected Intraperitoneally with Toxoplasma

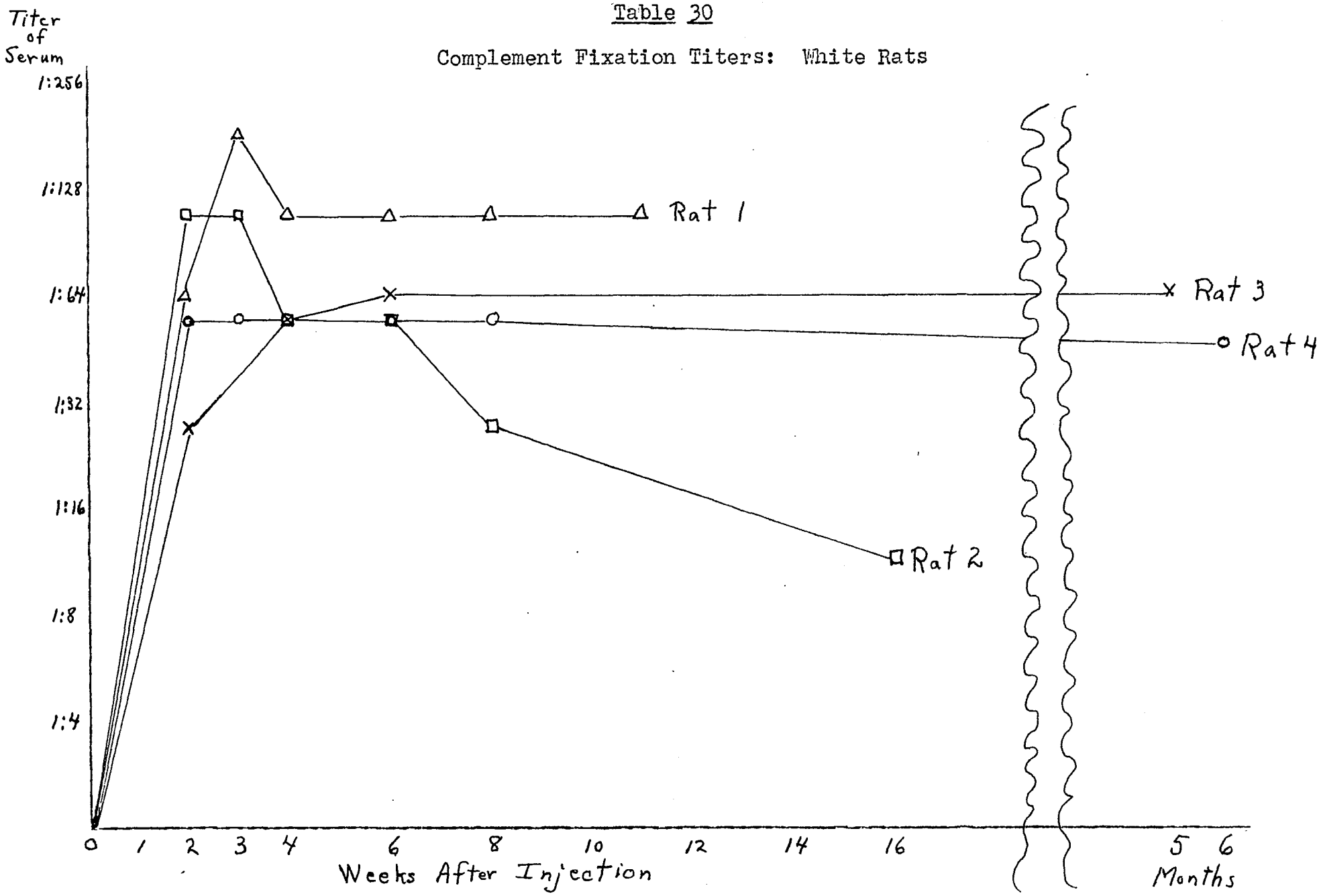
Rat	Weeks after Injection	Antigen or Control	Serum Dilution 1:4	1:8	1:16	1:32	1:64	1:128	1:256	a.c. 1:4
1	Normal	Tox. C.A. 3	0	0	0	0				0
		Normal "	0	0	0	0				
	2	Tox. "	0	0	0	0	0			0
		Normal "	4	4	4	4	4			
	3	Tox. "	0	0	0	0	0	0	0	0
		Normal "	4	4	4	4	4	4	2	
	4	Tox. "	0	0	0	0	0	0	0	0
		Normal "	4	4	4	4	4	3	2	
	6	Tox. "	0	0	0	0	0	0	0	0
		Normal "	4	4	4	4	4	3	3	
	8	Tox. "	0	0	0	0	0	0	0	0
		Normal "	4	4	4	4	4	3	3	
	11	Tox. "	0	0	0	0	0	0	0	0
		Normal "	4	4	4	4	4	3	2	
2	Normal	Tox. C.A. 3	0	0	0	0				0
		Normal "	0	0	0	0				
	2	Tox. "	4	4	3	1	0	0	0	0
		Normal "	4	4	4	4	3	3	2	
	3	Tox. "	3	1	0	0	0	0	0	0
		Normal "	4	4	4	4	4	3	2	
	4	Tox. "	2	1	0	0	0	0	0	0
		Normal "	4	4	4	4	3	3	2	
	6	Tox. "			0	0	0	0	0	0
		Normal "			4	4	3	3	2	(1:16)
	8	Tox. "	1	0	0	0	0	0	0	0
		Normal "	4	4	4	3	0	0	0	
	16	Tox. "	0	0	0	0	0	0	0	0
		Normal "	4	4	2	0	0	0	0	

dilutions. Rat 2 did show nonspecific fixation with sera obtained up to 8 weeks after infection, but only at the two-week bleeding was this significant. The 3-plus nonspecific fixation in a 1:16 dilution of this serum was the only example of significant nonspecific fixation which occurred in dilutions above 1:4 in a series of 8 rats. Three of these eight rats yielded no sera which fixed complement non-specifically, and sera from four other rats only did so in dilutions of 1:4.

Complement fixing titers for rats 1, 2, 3, and 4 are plotted in table 30. The titer of each serum was obtained by taking the highest dilution which gave a four-plus fixation and adding to this titer the amount of fixation in the next higher dilution. Thus, a serum which gave a 4-plus in a 1:32 dilution and a 3-plus in a 1:64 dilution was plotted at a point three-fourths of the distance between the 1:32 and 1:64 titers. Two weeks after injection these rats possessed titers between 1:32 and 1:128 dilutions. A peak appeared between two to four weeks, with titers about 1:64. Rats 3 and 4 had similar titers even at the 5th and 6th months, respectively; however, the titer of rat 2 began to fall at the eighth week and was only 2-plus in a 1:16 dilution at the sixteenth week. Rats 5, 6, 7, and 8 were bled at two-week intervals beginning four weeks after

Table 30

Complement Fixation Titers: White Rats

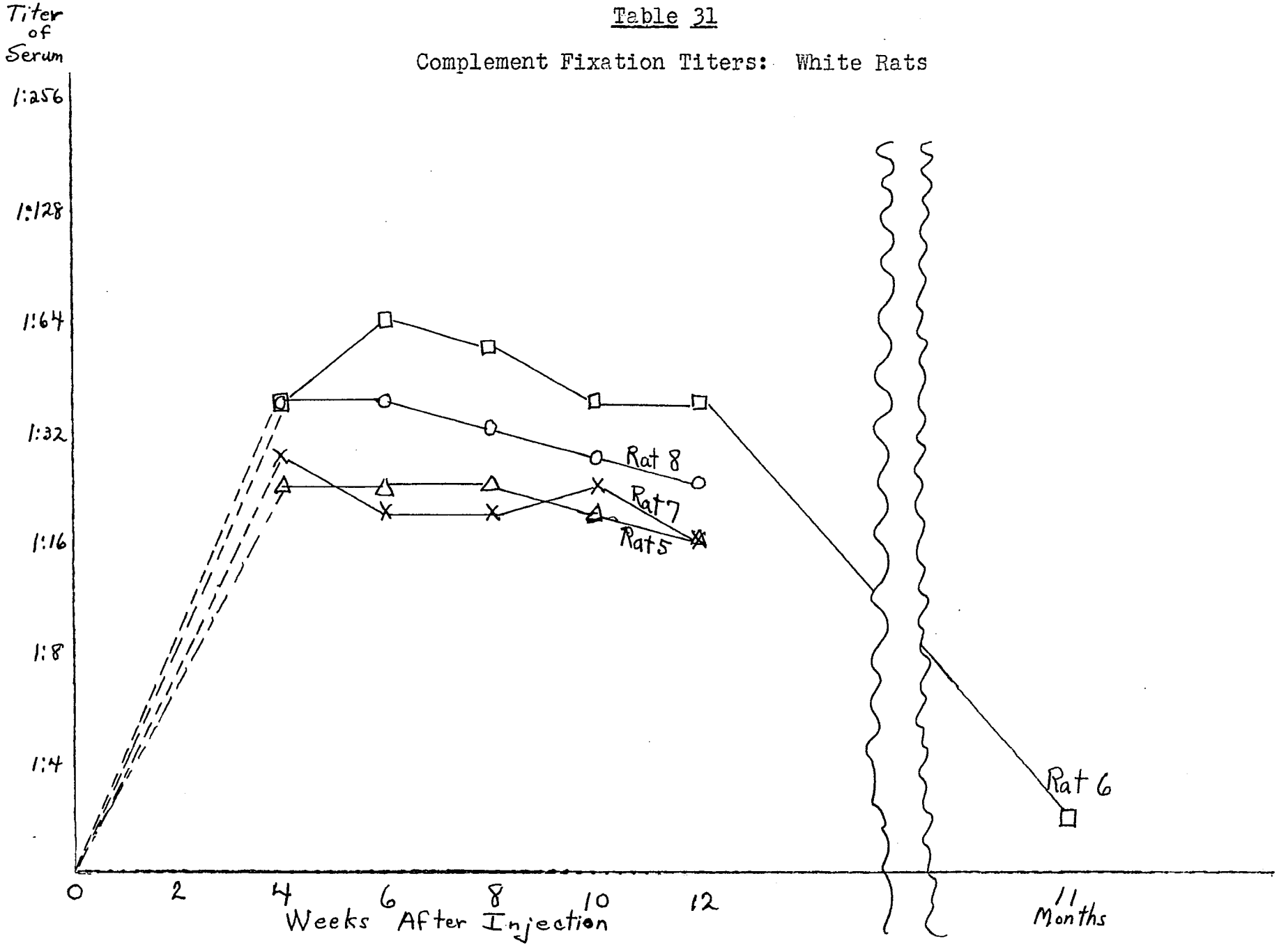


inoculation (Table 31). Titers of about 1:32 were found, and these were only slightly lower at 12 weeks. Rat 6 was sacrificed at 11 months, and toxoplasma were recovered from the brain. Serum obtained at this time fixed complement only to 2-plus in a 1:4 dilution. Neither the weight of the rat nor the amount of the inoculum appeared to affect the complement fixation titer that was attained or maintained. One additional rat was injected with 1.0 ml of a 10 per cent mouse brain, and bled at four and at seven days thereafter. Complement fixation with the four-day serum gave a 1-plus in a 1:4 dilution. The seven-day serum completely fixed complement in a 1:32 dilution, and gave no nonspecific reaction. Thus it appears that the complement fixing antibodies of white rats appear between the fourth and seventh days after injection, increase to a peak at 2 to 4 weeks, remain fairly strong for some weeks, and then gradually decrease. Normal white rat serum did not give any fixation during these studies.

Wild Rat Sera: Sera from nine wild rats captured in the Cincinnati area were tested for the presence of complement fixing antibodies. One rat specifically fixed complement to a dilution of 1:32 and therefore was either infected with toxoplasma at the time of capture or had previously had the disease. Another wild rat gave equivocal results, with

Table 31

Complement Fixation Titers: White Rats



an approximately 2-fold difference between fixation with the toxoplasma antigen and the control.

Fowl Sera: Six chickens and three pigeons were tested for complement fixing antibodies (Table 33). Chickens 2 and 4 received seven intraperitoneal injections of toxoplasma infected yolk sac and chorio-allantoic membrane. Sera obtained 30 days after the last injections contained no antibody. Similarly, chickens 5 and 6 received three intraperitoneal injections of living toxoplasma, and nine days after the last injection their sera contained no complement fixing antibody. Chicken 7 was sacrificed 90 days after intracerebral injection. Toxoplasma were recovered from the brain, but complement was not fixed by serum obtained at the time of sacrifice. Toxoplasma were recovered from the brain of chicken 8 when it was sacrificed 120 days after intracerebral inoculation, yet no complement fixing antibodies were present in the serum. Thus with six chickens no complement fixing antibodies were demonstrated. Three wild pigeons were bled from the heart and their sera were tested. None fixed complement. Pigeons 2 and 3 were each given 1.0 ml of a 10 per cent mouse brain suspension of the C.G. strain of toxoplasma. These pigeons survived, and 21 days after inoculation they were again bled. Pigeons 2 and 3 then had specific fixation titers of 16-fold. The R.H.

Table 33

Complement Fixation with Fowl Sera

Fowl	Regimen Employed	Days after Inoculation	Titer of Specific Fixation
Chicken 2	7 I-P* injections	30	0
" 4	" " "	"	0
" 5	3 " "	9	0
" 6	" " "	"	0
" 7	1 I-C** injection	90	0
" 8	" " "	120	0
Pigeon 1	—	Normal	0
" 2	C.G. Tox. I-P (1.0 ml)	"	0
		21	16
" 3	" " " "	Normal	0
		21	16

* Intraperitoneal injection of R.H. toxoplasma.
** Intracerebral " " " "

strain was found to be fatal to pigeons. These two avian species react differently to toxoplasma infection.

Guinea Pig and Rabbit Sera: The R.H. strain of toxoplasma is usually lethal to guinea pigs so that toxoplasma immune guinea pig serum is not easily obtained. Two normal guinea pigs were bled and then inoculated intracutaneously with 0.1 ml of a 10^{-3} dilution of C.G.. strain infected mouse brain. These guinea pigs were bled 19 and 33 days later. Neither guinea pig possessed complement fixing antibody at the 19th day, but at 33 days the titers were 64 and 128 for guinea pigs 1 and 2, respectively (Table 34).

During the course of testing human sera for neutralizing antibody, three rabbits were found to resist the intracutaneous injection of living toxoplasma. These rabbits were found to possess neutralizing antibody for toxoplasma. When tested for complement fixing properties resistant rabbits 2 and 3 exhibited more than 4-fold specific fixation (Table 34). All three rabbits gave a high degree of non-specific fixation, and the specific fixation titer of resistant rabbit 1 was therefore questionable but probably greater than 2-fold.

Placental Transfer of Complement Fixing Antibody: Two litters of rats born of toxoplasma infected mothers and one litter of guinea pigs born of a C.G. strain infected guinea pig

Table 34

Complement Fixation with Guinea Pig and Rabbit Sera

Animal	Sera	Titer of Specific Fixation
Guinea Pig 1	Normal	0
	19 days after C.G. tox. (I-D)*	0
	33 " " " " "	64
" " 2	Normal	0
	19 days after C.G. tox. (I-D)	0
	33 " " " " "	128
Rabbit 1	(Resistant to toxoplasma)**	?(>2)
" 2	" " "	> 4
" 3	" " "	> 4

* Intracutaneous injection of 0.1 ml of $10^{-3.0}$ mouse brain.

** Resistant to mouse brain employed in neutralization test.

were studied for possible demonstration of placental transfer of antibody (Table 35). Each group of young was bled at two intervals, and the two bleedings were tested simultaneously. Serum from one litter of white rats bled at five weeks of age gave a 2-plus fixation in the 1:4 and a 1-plus in the 1:8 dilution, and this small degree of fixation had disappeared at 11 ½ weeks. Serum from a three-week old litter gave better results, and fixed complement completely in a 1:8 dilution, but again this fixation capacity had also disappeared by the tenth week. Serum from three guinea pigs born from an infected mother fixed complement at 2-plus in both the 1:4 and 1:8 dilutions when tested at 1½ and 5 weeks of age. The low titers obtained in these three litters and the fact that the antibody was absent at the second bleeding of the rats, were accepted as evidences that the young had received their antibody passively from the mother, especially since these animals probably were not old enough to have produced antibodies of their own. These protective substances had either crossed the placenta or had been transferred through the mammary secretions.

Human Sera: Throughout a period of several years this laboratory has tested numerous specimens of human serum for their toxoplasma neutralizing antibody. These same sera were also tested for their ability to fix complement with toxoplasma antigens

Table 35

Placental Transfer of Complement Fixing Antibody

Mothers of Young Animals Tested	Age at Bleeding	Antigen or Control	Serum Dilutions 1:4	1:8	1:16	1:32	a.c. 1:4
Tox. Infected White Rats (Group 1)	Weeks 5	Normal	0	0	0	0	0
		Tox.	2	1	0	0	0
	11½	Normal	0	0	0	0	0
		Tox.	0	0	0	0	0
Tox. Infected White Rats (Group 2)	3	Normal	0	0	0	0	0
		Tox.	4	4	0	0	
	10	Normal	0	0	0	0	0
		Tox.	0	0	0	0	
Tox. Infected Guinea Pig (C.G. Strain of Tox.)	1½	Normal	0	0	0	0	0
		Tox.	2	2	0	0	
	5	Normal	0	0	0	0	0
		Tox.	2	2	0	0	

prepared from infected chorio-allantoic membranes. The sera were stored at dry ice temperatures until used. All sera were inactivated for 20 minutes at 37 C on either the same day or the day previous to testing for complement binding antibody. A total of 195 sera was tested with both the toxoplasma antigen and the control preparation. Specific and nonspecific fixation were encountered; therefore, it was necessary to analyze the results on the basis of the degree of specific fixation that occurred. This degree was expressed as the number of fold difference in fixation which the toxoplasma antigen gave over the control. Table 36 tabulates the degree of specific fixation and table 37 summarizes the distribution and per cent of nonspecific reactions among these sera. Thirty-three per cent of all sera tested reacted nonspecifically to some degree in a 1:4 dilution. In dilutions of 1:8, 1:16, and 1:32 these percentages were 14, 3, and 1, respectively. No correlation existed between nonspecific reactions and the degree of specific fixation of complement. On the basis of the results given in table 36, a serum was considered positive for toxoplasma antibodies if it gave greater than a 2-fold degree of specific fixation. The results with a serum which fixed complement 2-fold or less were considered indicative of a negative serum. Positive sera exhibited titers ranging from slightly more than 2-fold to greater than 32-fold, in which cases the endpoints were

Table 36

Degree of Specific Complement Fixation
(Human Sera)

Neutralization Results	Number of Sera	Specific* Complement Fixation Titer									
		0	<2	2	2-4	4	4-8	8	8-16	16-32	32 or >32
+	46	4	3	6	2	9	6	6	3	3	4
±	10	2	4	4	0	0	0	0	0	0	0
0	139	130	5	3	0	1	0	0	0	0	0

*Specific fixation is the number of fold difference in fixation between the toxoplasma antigen and the normal control.

Table 37

Distribution of Nonspecific Fixation among Human Sera

C.F. Result	Number of Sera Tested	Serum Dilutions				
		1:4	1:8	1:16	1:32	1:64
0	137	19	14	4	2	0
+	33	11	4	0	0	0
±	25	7	3	0	0	0
	195	37	21	4	2	0
				64		
				per cent		
		33	14	3	1	0

not reached.

A correlation between complement fixation and the neutralization test was possible (Table 38). All of 130 human sera were completely devoid of both types of toxoplasma antibody. Positive sera by both tests totaled 33. Eight serum specimens were equivocal when tested both ways (Table 40). Nine bleedings that were positive by neutralization were equivocal when tested for their ability to fix complement. Eight of these sera were from patients of more than 12 years of age in whom the disease had probably been present for some time (Table 39). This would not explain the results obtained with the serum from the 10-year old who had an 11 to 12 week interval between the onset of symptoms and the performance of the tests. Four sera with neutralizing antibody were negative when tested for fixation of complement (Table 39). Two of these were from adults who had had symptoms for 2 and 46 years, respectively. Thus, it appears that the complement fixing antibody may disappear from serum before the neutralizing antibody. Two infants of less than 1 month of age also were positive by neutralization and negative in the complement fixation test; yet it would be dangerous to suggest that the neutralizing but not the complement fixing antibody could cross the human placental barrier. Two sera that were negative by complement

Table 38

Correlation of Results of Neutralization and Complement Fixation Tests (Human Sera)

Result of Test		Number of Human Sera
Neutralization	Complement Fixation	
Negative	Negative	130
Positive	Positive	33
"	Equivocal	9
"	Negative	4
Equivocal	Equivocal	8
"	Negative	2
Negative	Positive	1
"	Equivocal	8
		(Total 195)

Table 39

Human Sera with Debatable Serologic Findings

Neutralization Test Results	C.F. Test Results	Interval between Onset of Symptoms and Test	Age	Additional Findings
+	±	20-30 yr.	34	Unilateral chorioretinitis
		12 "	12	Bilateral retinitis, convulsions
		10 "	15	" "
		6 "	33	" "
		3 "	20	" "
		11-12 wk.	10	Hepatosplenomegaly
		?	Adult	Mother of infant with pos. neut. test
		?	"	" " " " toxo. symptomatology
		?	"	Father "11 yr.old " pos. neut. test
+	0	46 yr.	50	Convulsions, cerebral calcification
		2 "	32	Bilateral chorioretinitis
		1 mo.	Infant	Head rolling, cerebral calcifications
		3 wk.	"	Hepatosplenomegaly, pigment (bile?) in serum

fixation were equivocal by neutralization, and eight sera that were equivocal for fixation were negative for neutralizing antibody (Table 40). These equivocal results might be expected in many disease conditions especially acute illnesses. One serum fixed complement specifically at least 4-fold, but did not neutralize toxoplasma. This serum was obtained from the father of a 3-week old son whose serum neutralized.

The specific complement fixation titers for all positive neutralizing sera were tabulated with regard to the interval of time between the onset of symptoms and the time of the test (Table 41). The highest complement fixing titers were obtained from patients who were tested within the first eighteen months after the appearance of symptoms. The three titers of 2-fold or less in the 0 to 6 month time interval were from children who were not old enough to produce their own antibodies. Patients with symptoms for long periods possessed low complement fixation titers, and in most cases had to be listed as having equivocal fixation. Nevertheless, one patient with a prolonged illness of 11 years fixed complement at least 8-fold.

Toxoplasmic infection is sometimes observed as a congenital encephalomyelitis, manifest either in utero or shortly after birth. Complement fixation tests were performed with the sera from a number of individuals who were suspected of having the

Table 40

Human Sera with Debatable Serologic Findings

Neutralization Test Results	C.F. Test Results	Interval between Onset of Symptoms and Test	Age	Additional Findings
±	±	10 yr. 1 " ? ?	31 14 Adult "	Unilateral chorioretinitis Brain tumor ? 5 mothers of offspring with neg. neut. test 1 mother " " " pos. " "
±	0	4 yr. 1 "	29 Infant	Unilateral neuroretinitis Head rolling, convulsions
0	±	5 mo. 4 " 6-10 wk. ? ? ? ? ?	12 38 25 Adult 8 12 Adult "	Muscle soreness, trichinosis Unilateral neuroretinitis Septic illness Unilateral retinitis of long duration " " " central choroiditis Mother of 12 yr. son with pos. sero. tests " " 2 " " " neg. " "
0	+	?	Adult	Father of 3 wk. son with pos. neut. test

Table 41

Relation of Complement Fixing Titer to Interval of Time Between Onset of Symptoms and Test

Time Interval	Specific Complement Fixation Titer					
	0	<2 or 2	2-4 or 4	4-8	>8-16	32 or >32
0-6 months	2	1	0	2	2	1
6-18 "	0	0	1	0	0	3
1½- 3 years	1	0	0	2	0	0
3-10 "	0	2	1	0	0	0
10-20 "	0	2	0	1	0	0
> 20 "	1	1	0	0	0	0
? Mothers*	0	2	6	6	4	0
? **	0	1	3	1	0	0

* Mothers of proven or suspected toxoplasmic children.

** Those with unknown time interval other than the group of mothers.

congenitally acquired illness, and when possible sera from members of their immediate families were also examined. The results of twenty such cases are listed in table 42. Fourteen patients and their mothers were tested. In nine instances positive serological tests were obtained from both mother and child. In five cases the child's complement fixing titer was higher than its mother's titer. The tests of two mothers were equivocal or negative by both fixation and neutralization while their children's tests were equivocal only by fixation and positive by neutralization. Of three other mothers' sera that gave positive neutralization tests, only one was positive by fixation. All three of the corresponding children were devoid of complement binding antibody, yet one of these children gave a positive neutralization reaction. Of four fathers who were tested the serum of one gave a positive neutralization test while that of a different father possessed only complement fixing antibody. Three siblings of these congenital cases were negative. Serum from a proven case of toxoplasmosis that was reported by Sabin (22) was positive by both tests. This serum sample was obtained six years after the onset of symptoms. Specimens were tested from five other mothers and all contained considerable antibody. Johansmann and Ruchman (14) recovered toxoplasma

Table 42

Human Serologic Findings: Toxoplasmosis of Probable Congenital Inception

Patient		Mother		Father		Sibling	
Age	Neut. Test	C.F. Titer	Neut. Test	C.F. Titer	Neut. Test	C.F. Titer	Neut. Test	C.F. Titer
3 wk.	+	0#	+	2	0	4		
6 "	+	4-8	+	4-8				
2 mo.	+	>32	+	16				
3 "	+	16	+	16				
4 "	+	8-16	+	8-16				
9 "	0	0	+	2	0	0	0	0
12 "	+	>32	+	4- 8				
13 "	+	>32	+	8-16				
2½ yr.	+	8	+	8				
3 yr.	+	8	+	8				
6 "	0	0	+	4				
10 "	+	2	+	2				
11 "	+	8	+	4	+	<2	0	0
12 "	+	2	0	2	0	0	0	0
5 hr.*			+	4- 8				
2 wk.p			+	8				
1 mo.†			+	8				
1 yr.**			+	4				
Infant***			+	4				
6 yr.❶	+	4						

\# Serum containing pigment (bile?)
* Post mortem: Toxoplasmic encephalitis, toxoplasma in lung cysts
p No autopsy: Convulsions, cerebral calcification, mental disturbances
† Proven case: Johansmann, R. J., and Ruchman, I., To be published
** Clinical diagnosis of toxoplasmosis in infant.
*** Post mortem: Toxoplasma in cysts
❶ Proven case: Sabin, A.B., (21)

from a child of one of these mothers by animal inoculation. Toxoplasma in cysts were observed at the post-mortem examination of the children of two other mothers with positive serologic tests. The fifth mother's infant was clinically diagnosed as a case of toxoplasmosis. In this series of congenital toxoplasmic patients the highest complement fixing titers occurred in the more recent infections, that is, among the younger children and their mothers.

In table 43 are reported the chief clinical findings of eleven congenitally transmitted and seven acquired cases of toxoplasmosis. Sera from all of these fixed complement specifically. Among patients with congenital toxoplasmosis, nine had cerebral calcification, ten had chorioretinitis or other signs of eye involvement, ten had hydrocephalus, one had microphthalmia, and five manifested convulsions or other signs of central nervous system involvement. Chorioretinitis and cerebral calcification were the important findings in this series. One or the other, or both, were present in each of these patients. This is in agreement with the observations of Ruchman (23) with respect to neutralization tests. Seven patients with toxoplasmosis acquired by modes other than in utero transmission are also reported in table 43. Patient Gar. was a mother who had been asymptomatic but who had given birth to children with typical

Table 43

Patients Whose Sera Fixed Complement

Inception	Patient	Age	Salient Clinical Festures
Congenital	Bla*	6 yr.	Hydrocephalus, cerebral calcification, chorioretinitis
	Car	3 mo.	" " ", macular degeneration
	Gan	2½ yr.	" " ", convulsions, chorioretinitis
	Gil	3 yr.	Central chorioretinitis
	Lan	4 mo.	Bilateral ", cerebral calcification, mental retardation
	McC	7 "	Hydrocephalus, cerebral calcification
	Mil	13 "	" " ", bilateral cataracts, convulsions, mental retardation
	Moc	11 yr.	Atrophic scar in perimacular region with pigment clumping
	Ric	6 wk.	Microphthalmia, congenital cataracts, cerebral calcification, hypoactive
	Tak	1 yr.	Hydrocephalus, bilateral chorioretinitis, cerebral calcification, convulsions, spasticity, strabismus
	Rus	2 mo.	Hydrocephalus, bilateral choroiditis, cerebral calcification
Acquired	Gar	Adult	Mother who gave birth to children with abnormalities
	Don	15 yr.	Bilateral chorioretinitis
	Dol	17 "	Older sister of girl with bilateral chorioretinitis
	Jla	38 "	Unilateral choroiditis, one hydrocephalic stillbirth
	Mea	12 "	Rash, fever, U.R.I., lymphadenitis, spleen?, myocardial damage
	Ros	15 "	Acute encephalitis of one wk., choroiditis
	Lyk	9 "	Prolonged encephalitis

* Proven case: reported by Sabin (22)

toxoplasmic abnormalities. She had probably had a mild or inapparent infection. Patient Dol. had also been without symptoms, yet her serum fixed complement. Patient Don. had bilateral chorioretinitis and patient Jla. had unilateral choroiditis and had previously been delivered of a hydrocephalic, dead foetus. A syndrome resembling Rocky Mountain spotted fever was found in patient Mea. In the 9 and 15 year old patients, Lyk. and Ros., encephalitis was the main clinical finding; however, patient Ros. did have a choroiditis. Among the patients with positive complement fixation reactions the following four toxoplasmic conditions have been manifested: (1) congenital encephalomyelitis; (2) acute encephalitis; (3) infection resembling Rocky Mountain Spotted fever; and (4) mild or inapparent infection.

In patient Rus. two serum specimens were tested for their ability to fix complement and it is noteworthy to point out that the titer dropped in the 2 month interval between bleedings. This was considered as additional evidence that the complement fixing antibody tends to decrease or disappear after the acute phase of the disease passes.

Most of the human sera used in this study were submitted because of the presence of one or more of the four cardinal clinical symptoms of toxoplasmosis, hydrocephalus, cerebral calcifications, chorioretinitis, and mental disturbances.

Some of the disease conditions associated with negative complement fixation are listed in table 44. Among the toxoplasma negative sera were at least two known positive syphilitic sera. Both of these reacted nonspecifically, one in the 1:4 dilution and the other to a 1:8 dilution, but neither gave a false positive complement fixation for toxoplasmosis.

DISCUSSION:

The inoculation of toxoplasma into the yolk sac of developing chick embryos invariably caused death of the embryo within four to ten days after inoculation. This was found to be true with two strains of toxoplasma. One of these strains had been well adapted in white mice and uniformly killed mice. The other, of more recent isolation, was not as well adapted to mice, and only an occasional mouse died as a result of infection with this strain. Levaditi, et al, (1) and Wolfe, Cowen, and Paige (2) inoculated fertile eggs with toxoplasma, yet their eggs hatched. Chicks hatching from such eggs died after several days, and were found to be heavily parasitized. These investigators used recently isolated strains for egg inoculation. Since the C.G. strain that was used in some of the experiments during these investigations was only in its ninth

Table 44

Disease Conditions with Negative Complement Fixation Reaction

Microcephaly

Hydrocephalus, chorioretinitis

Unilateral chorioretinitis

Bilateral chorioretinitis

Anencephaly

Histoplasmosis

Hodgkin's disease

Torulosis

Hepatosplenomegaly

Subdural hematoma

Miliary tuberculosis

Familial demyelinizing encephalopathy

Familial amaurotic idiocy

Atresia of aqueduct of Sylvius with hydrocephalus

Syphilis

Acrodynia

animal passage the chick embryo cultivation with resultant death was at some disagreement with the results of the above authors. This difference may represent strain variation.

The embryonated hen's egg may be an adjunct for the primary isolation of toxoplasma although the probability is that for initial recovery of strains the mouse is still the animal of choice.

The cultivation of toxoplasma in the fertile egg is an easily handled and practical laboratory method for the preparation of antigenic materials for serological studies. Infected chorio-allantoic membranes, when treated by freezing and thawing, supplied reliable complement fixing antigens although it was necessary to test all sera simultaneously with normal membranes as a control. With such antigens the white rat has been found to be a much more suitable laboratory animal than the rhesus monkey. White rats are also advantageous in being inexpensive and easily handled. The course of infection in rats appears in many ways to resemble that in man.

Complement fixing antibodies have been demonstrated in man and in all animals tested with the exception of chickens. However, nonspecific reactions were frequently encountered, particularly in man and the monkey. During these experiments

the nonspecific reaction was controlled by the use of normal preparations of chorio-allantoic membrane; however, alternative or additional methods are available but have not as yet been tried. During the preliminary experiments with rabbit brain antigens, the method of Casals and Palacios (19) of inactivating antisera at 65 C did not increase the degree of specific fixation. This method might eliminate many of the nonspecific reactions encountered with human and other sera with toxoplasma antigens prepared from embryonic egg tissues. De Boer and Cox (24) have succeeded in decreasing or removing the nonspecific reactions from some virus systems by extraction of the complement fixing antigen with benzene. The application of this procedure might enhance the value of toxoplasma complement fixation tests.

The results obtained by complement fixation reactions during these experiments have been in agreement with the original hypothesis of Warren and Sabin (12), that the complement fixing antibody and the neutralizing antibody are different entities. Complement fixing antibody tends to decrease or disappear before the neutralizing antibody. Furthermore the complement fixing antibody is not destroyed by heating at 56 C for 30 minutes, but neutralizing antibody is destroyed under such conditions.

Because of the ease of preparation of toxoplasma chorio-allantoic membrane antigens, and performance of the complement fixation test with such antigens, the value of the complement fixation reaction in epidemiological and diagnostic studies should not be underestimated. This is particularly true since a high complement fixation titer is much more indicative of recent or active infection than is the mere presence of neutralizing antibodies.

SUMMARY:

Two strains of toxoplasma were successfully propagated in the embryonated hen's egg. One strain represented a well adapted laboratory strain; the other was nine animal passages away from a fatal human case. Essentially the same uniform results were obtained. Death occurred in from 4 to 10 days after inoculation. Visible lesions were present on the chorio-allantoic and amniotic membranes.

Titrations in mice revealed that the chorio-allantoic membrane, the embryo proper, and the yolk sac contained numerous parasites. The egg fluid titers were lower. The chorio-allantoic membrane titers approximated those obtainable with the infected mouse brain.

Comparative toxoplasma titrations in chick embryos and mice gave similar results.

The age of the fertile egg, the temperature of incubation, and the route of inoculation were important factors that were investigated.

The repeated passage of toxoplasma through eggs resulted in no modification of their pathogenicity for animals.

Serum neutralization tests were successfully performed in chick embryos.

Infected chick embryo tissues maintained viable parasites at 4 C for considerably longer storage periods than did infected mouse brains.

Infected chick embryo tissues, particularly the chorioallantoic membrane, were rich sources for complement fixation antigens. Sera from rhesus monkeys, white rats, wild rats, pigeons, guinea pigs, chickens, rabbits, and man were tested for complement fixing antibody. The white rat was found to be a suitable and easily handled laboratory animal for the serologic study of toxoplasma. The results obtained with human sera correlated well with those of the serum neutralization test and with the known chief clinical findings.

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