TABLE II.
Two-Stage Assays: Dilution of Various Plasmas Is That in the 2 cc of Incubation Mixture Containing Calcium and Thromboplastin. Units of Prothrombin Are Calculated in Terms of Original Plasma or Total Plasma When Aged Plasma Is Included. Amount of Serum Is That Added to the 2 cc Incubation Mixture. The Very Low Yields of Thrombin Give Clotting Times Too High for Accurate Determination.

Plasmas and dilutions		Serum, cc	Yield of thrombin, units per cc of plasma
Untreated	1:100	0	490
None		0.1	< 5
Zymin	1:40	0	Slight trace
"	1:40	0.1	280
NH_3	1:40	0	<20
"	1:40	0.1	290
Zymin	1:80 + NH_3 1:80	0	320
Aged	1:40	0	<20
"	1:80	0.1	300
"	1:80 + Zymin 1:80	0	300
"	1:80 + NH_3 1:80	0	<20

N.B. Units of prothrombin are determined in terms of yield of units of thrombin.

TABLE III.
Tests for Complement Activity: 1 cc Sensitized Sheep Cells Added to Each of the Following.

Components present	Hemolysis
.1 cc serum + 0.1 cc saline	Complete
.1 " untreated plasma + 0.1 cc saline	"
.2 cc zymin plasma	None
.2 " NH_3 "	"
.2 " aged "	"
.1 " zymin " + 0.1 cc NH_3 plasma	Partial
.1 cc zymin plasma + 0.1 cc aged plasma	"

bin while leaving abundant prothrombin which is highly reactive in the presence of the proper conversion factors, makes it somewhat doubtful whether any method of assay yet devised measures a single substance, prothrombin. The foregoing experiments point to a complex rather than a simple prothrombin conversion factor.

The inactivations of the third and fourth components of complement by zymin and by ammonia respectively are regarded as fairly specific, being in fact the bases for definition of these components. To regard the blood coagulation system and complement as closely related appears to be the point of view most likely to direct efforts toward increasing knowledge of both.

Summary. Inactivation of the complement of plasma by aging or by treatment with zymin or with ammonia blocks conversion of prothrombin to thrombin while leaving ample reactive prothrombin.

16213

Cultivation of Toxoplasma in Embryonated Egg. An Antigen Derived from Chorioallantoic Membrane.

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Neutralization[1] and complement-fixation[2] tests have been used for establishing the diagnosis of toxoplasmosis in man; however, neither method is entirely satisfactory. The

1 Sabin, A. B., and Ruchman, I., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 1.

2 Warren, J., and Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 11.

former requires the use of living parasites and test animals, whereas the rabbit brain antigen, generally used in the latter, gives non-specific reactions under certain conditions. The present report describes the successful cultivation of toxoplasma parasites in embryonated eggs together with a method for preparing complement-fixing antigen from infected chick embryo tissue. Preliminary diagnostic data obtained using this antigen in tests with sera of patients are included.

Materials and Methods. (a) *Strain of Toxoplasma and Its Maintenance in Embryonated Eggs.* The RH strain of toxoplasma of human origin[3] was employed in the present studies. Infected mouse brain material was inoculated into embryonated eggs and the agent subsequently was maintained by serial passage in the avian host. For this purpose the brains of those embryos which appeared moribund 7 to 8 days after inoculation were removed, and a 10% suspension of tissue, partially clarified by light centrifugation, was injected in 0.1 cc amounts into the chorioallantoic sacs of normal embryos. Impression films, prepared from the brain tissue of selected embryos, were stained by Giemsa's method and the presence of toxoplasma was verified before passage was performed.

(b) *Preparation of Complement-fixing Antigen.* Chorioallantoic membranes bearing macroscopic lesions were harvested from 12 or more eggs on the seventh day after inoculation. These were pooled, ground in a mortar with sterile alundum and a 10% suspension was prepared by adding physiological saline solution buffered at pH 7.4. This crude suspension was frozen and thawed 3 times and then clarified by centrifugation at 3,500 r.p.m. for 15 minutes in an angle machine. The supernatant fluid, after addition of sufficient merthiolate to bring the concentration to 1:10,000, constituted the antigen. This was generally stored at −20°C until used. Normal 16-day-old chick embryos were similarly treated to prepare an antigen for use as control material.

(c) *Specific Immune Sera.* Immune sera were obtained from rhesus monkeys and from guinea pigs convalescent from infection with toxoplasma. Monkeys were infected by the subcutaneous inoculation of 0.1 cc of mouse brain suspension containing approximately 10,000 to 100,000 minimal lethal doses of the organism as estimated by intracerebral titration in mice. Guinea pigs were inoculated intraperitoneally with 100 to 1,000 minimal lethal doses of toxoplasma similarly estimated. Both monkeys and surviving guinea pigs were bled for immune serum approximately 30 days after inoculation.

Sera from a number of patients who had been suspected of having a toxoplasma infection were available for study. These sera had been stored at −20°C for from a few days up to 3 years.

(d) *Neutralization Tests.* The presence in serum of neutralizing antibodies against toxoplasma was determined by the method of Sabin and Ruchman.[1] In this procedure mixtures of serum and infectious mouse brain suspension are inoculated intracutaneously into rabbits and the resultant lesions are read on the fourth to seventh day.

(e) *Complement-fixation Tests.* Tests for complement-fixing antibodies were performed in the following manner: Tubes containing 0.25 cc amounts of the appropriate dilutions of the serum to be tested received 0.25 cc of antigen (4 units) and 0.5 cc of fresh diluted guinea pig serum containing 2 units of complement as determined by preliminary titration in the presence of four units of antigen. These were incubated overnight at 5°C. The hemolytic system, consisting of 0.5 cc of an equal mixture of a 3% suspension of washed sheep erythrocytes in saline and diluted amboceptor (2 units), was then added to the tubes. The mixtures were incubated at 37°C for a half hour and then read in the usual manner. In addition to the usual controls, each serum was tested with normal antigen employed at a dilution comparable to that of the toxoplasma antigen.*

Experimental. (a) *Cultivation.* The introduction of toxoplasma into the chorioallantoic, amniotic, or yolk sacs almost invariably re-

[3] Sabin, A. B., *J. A. M. A.*, 1941, **116**, 801.

* All complement-fixation tests reported in this paper were performed by Mr. M. J. Snyder of this department to whom the authors wish to express their gratitude.

TABLE I.
Distribution of Toxoplasma in Embryonic Tissues.

Egg passage No.	Days after infection	Intracerebral infectivity titer of tissue for mice				
		Emb. brain	Emb. torso	Allant. memb.	Allant. fl.	Yolk sac
1	7	$10^{-4.4}$	$10^{-4.5}+$	$10^{-4.5}+$	—	$10^{-4.0}$
2	7	$10^{-2.6}$	$10^{-3.5}$	$10^{-3.3}$	—	$10^{-1.5}$
3	8	$10^{-4.4}$	$10^{-3.5}$	—	$10^{-1.8}$	$10^{-2.5}$
3A	7	—	—	$10^{-5.5}$	—	—
5	8	$10^{-4.6}$	$10^{-3.5}$	$10^{-3.5}$	—	$10^{-2.5}$
11	7	$10^{-4.5}$	$10^{-4.0}$	$10^{-3.8}$	—	$10^{-2.0}$

Embryos were 10 days old at time of inoculation and were incubated at 35°C after infection.

sults in a fatal infection. Growth of the parasite occurs equally well in embryonated eggs which are 6 to 12 days old when inoculated; embryos succumb between the seventh and tenth days after infection with practically all dying on the eighth day. Multiplication of the organism occurs equally well in eggs incubated at 35° or 37.5°C.

Toxoplasma are demonstrable microscopically in all tissues of infected embryos. Tissues and fluids of inoculated eggs have infective titers of $10^{-2.0}$ to $10^{-5.5}$ when tested in mice. However, as indicated in Table I, the highest concentrations of the parasites usually are found in the embryo and allantoic membrane.

The most striking pathological lesions in the infected embryonated eggs are large yellowish-gray nodules, scattered throughout the allantoic membrane (Fig. 1). These may be 2 mm in diameter and are frequently visible through the unbroken shell upon transillumination. The embryos themselves are deeply hemorrhagic and occasionally show nodular lesions in the viscera or the skin. The nodules on microscopic examination consist of a central area of necrosis surrounded by a zone of mononuclear leukocytes and contain many toxoplasma scattered throughout the necrotic and infiltrated portions of the lesion.

(b) *Complement-fixing Antigen.* Antigens prepared from infected chorioallantoic membrane were compared in complement-fixation tests with antigens derived by a similar procedure from infected rabbit and mouse brain tissues.[2] Antigens from all 3 sources had titers of 1/8 to 1/16 when tested with 2 units of antibody. The complement-fixing material in the chick embryo preparation had the characteristics of a specific soluble substance since it was separable from the intact organism; data presented in Table II show that no diminution in activity resulted when chick embryo antigens were passed through a Seitz filter or were centrifuged at 14,000 r.p.m. in an angle machine for one hour. Moreover, the antigens are relatively stable; complement-fixing activity was not reduced by lyophilization nor by storage at −20°C for 3 months.

(c) *Preliminary Evaluation of Complement-fixation for Diagnosis of Human Toxoplasmosis.* A number of stored frozen samples of serum were available which had been submitted from adults and children suspected of having toxoplasmosis, all of which had been previously tested for neutralizing antibody. In addition, control sera obtained from "normal" and from syphilitic adults were examined. The above sera were tested for complement-fixing antibodies employing antigen prepared from chorioallantoic membrane. Four of the 11 "normal" sera and 3 of the 14 sera with positive Wassermann reactions fixed comple-

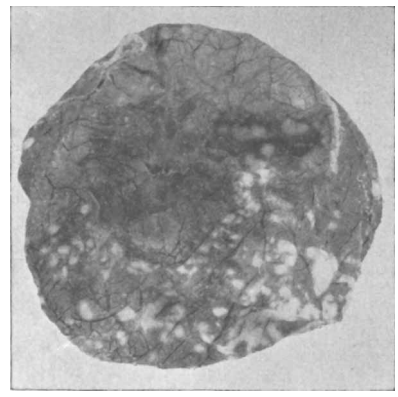

FIG. 1.
Chorioallantoic membrane infected with toxoplasma.

TABLE II.
Complement-Fixation Reactions with Toxoplasma.

Serum used	Serum dilution	Toxoplasma chorioallantoic membrane antigen: Crude suspension 1:2	1:4	1:8	1:16	After desiccation and dehydration 1:2	1:4	1:8	1:16	Seitz filtrate of antigen 1:2	1:4	1:8	1:16	14,000 rpm/60′ 1:2	1:4	1:8	1:16	Normal antigen Crude suspension 1:2
Toxoplasma immune guinea pig	1:25	4+	4+	4+	0	4+	4+	4+	0	4+	4+	+	0	4+	4+	4+	0	0
	1:50	4+	4+	4+	0	4+	4+	3+	0	4+	4+	0	0	4+	4+	3+	0	0
	1:100	4+	4+	+	0	4+	4+	3+	0	4+	4+	0	0	4+	4+	0	0	0
	1:200	4+	3+	0	0	4+	4+	0	0	+	2+	0	0	+	3+	+	0	0
	1:400	0	0	0	0	2+	3+	0	0	0	0	0	0	0	0	0	0	0
Normal guinea pig	1:25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

ment at dilutions of 1/4 to 1/8 with both toxoplasma and normal egg antigens. All of the "normal" sera which reacted in these tests, however, were obtained from individuals immunized with vaccines of egg origin, and fixation of complement by mixtures of normal egg antigen and sera from vaccinated or syphilitic persons is well known.[4,5] By contrast, sera from 47 adults with clinical diagnoses of chorio-retinitis, uveitis, iritis, or visual impairment, all of unknown etiology, were examined. None of these sera had significant amounts of neutralizing or complement-fixing antibody of toxoplasmosis. Tests were also made on sera from 7 children, aged from 6 weeks to 2½ years, on whom a diagnosis of toxoplasmosis was entertained because of 2 or more of the following manifestations: chorio-retinitis, hydrocephalus, cerebral calcification, and mental retardation. Both complement-fixation and neutralization tests were negative with 5 sera and both were positive with the remaining two (infants A and B in Table III). The diagnosis of toxoplasmosis was proved in infant A by recovery of the parasites in inoculated animals and their microscopic demonstration in human autopsy material.

Sera from the healthy mothers of infants A and B both contained neutralizing and complement-fixing antibodies for toxoplasma (Table III). Both types of test were also positive on sera from a third mother who delivered a child with congenital cataracts; serum from the child was not available for study. Negative results were obtained in both types of test with sera from 2 families, mother and child, in which the infant was suspected of having toxoplasmosis on clinical grounds.

The serological results obtained in the case of a 30-year-old male with a peculiar syndrome are worthy of mention. This man (patient F in Table III) had a nodular dermatitis, chorioretinitis, weakness, weight loss, eosinophilia and low grade fever, all of approximately 2 years duration. His serum gave a complement-fixing titer of 1/128 and a strongly positive neutralization test. Repeated attempts to recover toxoplasma from cutaneous lesions,

[4] Wertman, K., *J. Lab. Clin. Med.*, 1945, **30**, 112.

[5] Smadel, J. E., Warren, J., and Snyder, M. J., *J. Bact.*, 1947, **54**, 77.

TABLE III.
Positive Serological Reactions of Patients Having Clinical Evidence of Toxoplasmosis.

Patient	Clinical manifestations	Test	
		Neutralization*	Complement-fixation (Titer)
A	Fatal case of toxoplasmosis in infant	Strongly pos.	1:32
B	Infant with chorioretinitis, cerebral calcification and mental retardation	Positive	1:128
C	Healthy mother of infant A	Strongly pos.	1:128
D	Healthy mother of infant B	Positive	1:128
E	Healthy mother of infant with congenital cataracts	,,	1:16
F	Adult with cutaneous nodules, chorio-retinitis and fever	,,	1:128

* Neutralization by the test serum of a 1:100 dilution of toxoplasma-infected tissue when the mixture is injected intracutaneously in the rabbit is regarded as a positive reaction; neutralization of a 1:20 dilution as a strongly positive.

blood, and sternal marrow gave negative results. Because of these serological findings, the patient was tested for cutaneous reactivity to toxoplasma material. For this purpose, 1/100 and 1/1000 dilutions of standard complement-fixing antigen and a 1/1000 dilution of normal egg antigen prepared in a similar fashion were injected intracutaneously in 0.1 cc amounts. Although there was no immediate reaction at any of the sites, both of the areas receiving the toxoplasma antigen developed erythema and induration at 24 hours which persisted for several days; the lesion at the site of the 1/100 dilution was 1½ x 2 x 0.1 cm and at the 1/1000 dilution was 1 x 1 x 0.1 cm. No reaction was elicited at the site of the injection of normal egg material.†

Summary. Inoculation of embryonated eggs with toxoplasma resulted in a generalized parasitic disease of this host with a fatal termination. The agent could be maintained by serial passage of embryo brain. Chorioallantoic membranes from infected eggs contained a stable, specific soluble antigen which fixed complement with toxoplasma immune animal serum. Human sera from both proven and suspected cases of toxoplasmosis which were known to contain neutralizing antibody against the parasite also fixed complement with chorioallantoic membrane antigen.

† These studies were performed in collaboration with Drs. A. J. Brennan and T. Brown, Mount Alto Hospital, Washington, D.C., and will be reported in detail elsewhere.

16214

Influence of Oxophenarsine on Hypoglycemic Action of Insulin.

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Noting the results obtained by Barron and Singer[1] in inhibiting the activity of certain enzymes with arsenoxides and their use of this reaction as a test of the essential SH groups of such enzymes, the author thought it of interest to incubate insulin under sterile conditions with the arsenoxide, oxophenarsine (3-amino-4-hydroxyphenyl arsenoxide hydrochloride). While enzyme workers assume that reaction is only with free SH groups, it seemed possible that a part of the arsenoxide might reduce the S-S linkages of insulin to –SH,

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1 Barron, E. S. G., and Singer, T. P., *Science*, 1943, **97**, 356.