old, moldy, normal monkey sera and tested them against toxoplasma, but with completely negative results.

Neutralizing antibodies in other animals. Fresh sera of normal rabbits, mice, cats, and dogs contained no antibody nor was any found in these animals after recovery from experimental infection with toxoplasma (the studies on the sera of cats and dogs were carried out in association with Dr. Joel Warren).

Lability of toxoplasmic neutralizing antibody in human sera. Although a number of scattered tests had indicated that the toxoplasmic neutralizing antibody was probably as labile in human as in monkey sera, it was desirable for practical and other reasons to determine some of the limits of this lability. Serum, known to contain neutralizing antibody, was obtained from an 18-month-old infant with congenital toxoplasmosis[5] and divided into several portions. Part was immediately frozen and stored in a solid CO_2 refrigerator, part was tested fresh, and other portions were tested after storage at room temperature (about 24° to 26°C) or in an ordinary refrigerator (about 4° to 5°C). The data illustrated in Chart II reveal that the same serum which yielded strongly positive results when tested fresh or after being frozen at about 70°C for 14 weeks gave an equivocal result after 3 days at room temperature or 1 week in the refrigerator and a distinctly negative result after 1 week at room temperature. The neutralizing antibody in the human serum was also inactivated by heating at 56°C for 30 minutes.

Summary. The toxoplasma neutralizing antibody was found to be so labile even at temperatures of about 5°C, that it could disappear after one to 2 weeks of storage in an ordinary refrigerator. The antibody effect was destroyed by heating at 56°C for 30 minutes and could not be restored by the addition of fresh complement. The antibody could be preserved for months at the low temperature provided by solid CO_2 in an insulated box. Antibody was absent in the fresh sera of rhesus monkeys before infection with toxoplasma but appeared within one to 2 weeks after infection and persisted for at least 14 months which is the limit of observation thus far. This persistence of neutralizing antibody was not associated with persistence of toxoplasma in the monkeys, since none could be found in tests on a large number of tissues from 3 monkeys, 14, 17, and 26 weeks after infection. No neutralizing antibody was found in the fresh sera of normal rabbits, mice, cats, or dogs, nor did these animals develop any appreciable antibody after recovery from experimental infection with toxoplasma.

5 Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 6.

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Toxoplasma Neutralizing Antibody in Human Beings and Morbid Conditions Associated With It.

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By actual demonstration of the organisms in the tissues, by microscopic and animal inoculation methods, toxoplasma have already been proved to be responsible for the following morbid conditions in human beings: (a) congenital encephalomyelitis becoming apparent in the newborn period or *in utero*,[1,2] (b) acute encephalitis in childhood,[3] and (c) a spotted-fever-like syndrome associated with pneumonitis.[4] The fact that mothers who had given birth to infants with proved

1 Wolf, A., Cowen, D., and Paige, B. H., *Am. J. Path.*, 1939, **15**, 657; *Science*, 1941, **93**, 548.

2 Paige, B. H., Cowen, D., and Wolf, A., *Am. J. Dis. Child.*, 1942, **63**, 474.

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

congenital toxoplasmosis, themselves gave no definite history of illness suggested that mild, subclinical, or even inapparent infections with toxoplasma may occur.

When the special properties of the toxoplasma neutralizing antibody were discovered and convincing evidence was obtained that the rabbit skin neutralization test yielded reproducible results,[5] a study was undertaken to determine, (a) the incidence and significance of this antibody in human beings, and (b) whether or not certain morbid manifestations in which a toxoplasmic etiology might be suspected, were associated with a high incidence of positive neutralization tests. The following procedure was adopted for the test:

Animals. Clear-skinned (*i.e.*, without ingrown hair), well-nourished rabbits, weighing about 3 kg gave the best results. The hair was clipped with an Oster animal clipper (000 or 0000). Among the 105 rabbits obtained from 2 different dealers (Ohio and New York) and used for neutralization tests in the past year, we encountered 5 which were naturally immune (this incidentally is an interesting indication of the possible incidence of nonfatal toxoplasmic infection among rabbits in certain regions). A naturally immune rabbit is recognized by the fact that small, rapidly regressing or insignificant skin lesions develop with the control mixtures and the animal survives at a time when other rabbits inoculated simultaneously with the same toxoplasma suspension develop the usual progressive lesions and die.

Serum. Whenever possible the serum was used on the day of bleeding. Sera which had been in the refrigerator overnight or in the mail for 24 to 48 hours (during the winter months) have also yielded positive results. The unused portion of each serum was frozen rapidly in a lusteroid container and stored in an insulated box containing solid CO_2 to permit repetition of the test whenever necessary. Serum which could not be tested within 24 hours of bleeding was similarly frozen and stored in the CO_2 refrigerator.

Toxoplasma suspension. The R. H. strain of toxoplasma, which had undergone many mouse passages since it was isolated in 1939 from a fatal case of human encephalitis,[3] was used in these tests. The brains of mice succumbing 4 days after intracerebral inoculation were examined by means of Wright-stained films to make certain of the presence of large numbers of toxoplasma and the absence of bacteria. At least 2 such brains were ground in a mortar without an abrasive and enough Tyrode's solution was added to make a 10% suspension. This suspension was then allowed to sediment spontaneously for 30 minutes in an ordinary refrigerator. From the opalescent supernatant liquid which is regarded as the 1:10 dilution, the 1:50, 1:500 and 1:5000 dilutions were prepared using Tyrode's solution as the diluent. Mixtures consisting of 0.15 cc of undiluted serum or Tyrode's solution (for the control) and 0.15 cc of each of the above dilutions thus yielded final dilutions of toxoplasma-containing mouse brain suspension of 1:20, 1:100, 1:1000, and 1:10,000. After thorough shaking these mixtures were left at room temperature for 30 minutes before inoculation. The back of each rabbit was marked off into 20 squares with an indelible pencil to accommodate all the dilutions of the Tyrode's solution control and 4 sera. 0.2 cc of each mixture was injected intracutaneously.

Interpretation of results. All human sera produce erythema and oedema in the rabbit's skin within 24 hours, but this reaction has usually disappeared by the 4th day when the toxoplasma lesions begin to appear. The toxoplasma skin lesions are raised, indurated and the ones resulting from the larger concentrations undergo central necrosis; they are fully developed by the 7th or 8th day, when a record is made by tracing on transparent paper the size of each lesion and of the area of necrosis. The skin lesions begin to blanch after the 8th day and the rabbits with very rare exceptions die of a generalized toxoplasmosis between the 9th and 12th days.

With this strain of toxoplasma and with the infected mouse brain suspension pre-

[4] Pinkerton, H., and Henderson, R. G., *J. A. M. A.*, 1941, **116**, 807.

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

TABLE I.
Criteria Used in Interpretation of Toxoplasma Neutralization Tests.

Category of unknown serum	Relative size of skin lesions yielded by mixtures containing various dilutions of infected mouse brain suspension			
	1:20	1:100	1:1000	1:10,000
Negative	++++	++++	+++	++ or +
Negative	++++	++++	+++	0
Equivocal (Repeat test)	++++	+++	++ or +	0
Positive	++++ or +++	+++ or ++	0	0
Strong positive	++	0	0	0

pared according to the above directions, distinct lesions appeared in all susceptible rabbits at all sites inoculated with the 1:20, 1:100, and 1:1000 dilutions of the control mixtures while the 1:10,000 dilution produced lesions less regularly but more often than 75% of the times. The mixtures with most of the distinctly negative human sera yielded skin lesions which were somewhat larger than the Tyrode's solution controls. Table I indicates the criteria used for classifying the sera. Repeated tests on the same specimen of a positive serum (preserved in the frozen state) revealed the reliability of these criteria, and tests on repeated bleedings from the same individual gave ample evidence of the reproducibility of the results.

Results of tests on human sera. Toxoplasma neutralization tests were performed on sera from 151 selected individuals. The following considerations guided the selection of individuals: (a) the presence in infants or children of one or more of the manifestations that may now be considered to constitute the clinical tetrad of congenital toxoplasmosis, *i.e.*, (1) hydrocephalus or microcephaly, (2) cerebral calcification, (3) chorioretinitis, (4) disturbances of nervous function such as convulsions, psychomotor retardation, etc.; (b) the mothers and whenever possible other members of the families of such infants and children; (c) mothers who gave birth to hydrocephalic or microcephalic infants who were either stillborn or died shortly after birth; (d) mothers who gave birth to anencephalic monsters; (e) older children and adults exhibiting chorioretinitis of unknown etiology and resembling the type encountered in infants with congenital toxoplasmosis; (f) children who were hospitalized in Cincinnati during the summer and autumn of 1939 because of an encephalitis bearing a clinical resemblance to the proved fatal case of toxoplasmic encephalitis which occurred at that time;[5] (g) certain unexplained encephalopathies; (h) a large miscellaneous group made up of normal individuals, and children and adults taken at random from the hospital wards or tested because they had febrile illnesses of unknown etiology, obscure disturbances of the nervous system, congenital cataracts or other disturbances of the eyes, etc.

The sera of 92 of the 151 individuals were negative. The 59 individuals whose sera neutralized toxoplasma were derived from all the groups listed above. In the group of infants and children presenting various disturbances which used to be ascribed to birth injury, hemorrhage, congenital deformity, etc., it was found that the incidence of neutralizing antibodies was high in the patients and their mothers only when certain clinical manifestations occurred together (Table II). Thus, when cerebral calcification or chorioretinitis in the macular region or both were associated with hydrocephalus or microcephaly or with convulsions and psychomotor retardation, distinctly positive neutralization tests were obtained in 10 of the 13 infants or children and in 8 of 10 of their mothers. Specific toxoplasmic complement fixation reactions[6] were obtained in repeated tests on different specimens of serum from one of the patients (with suspected congenital toxoplasmosis, first group Table II) and his mother who were regularly negative in the neutralization test. By comparison it is important to note that

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

TABLE II.
Toxoplasma Neutralizing Antibodies in Human Beings with Certain Obscure Diseases.

Clinical manifestations		No. of individuals with toxoplasma neutralizing antibodies among: Patients	Mothers of patients
Cerebral calcification and/or chorioretinitis in macular region associated with hydrocephalus, microcephaly and/or convulsions, psychomotor disturbance, etc. Age group—birth to 15 yrs		10/13	8/10*
Hydrocephalus or microcephaly *without* cerebral calcification or chorioretinitis. Age group—9 wks to 5 yrs		0/10	1/4
Convulsions, psychomotor disturbances, etc., *without* cerebral calcification or chorioretinitis Age group—birth to 5 yrs		1/9	1/2
Chorioretinitis of unknown etiology resembling that seen in congenital toxoplasmosis	*Probably congenital* Disturbance of vision first noted at 5 to 6 yrs. Age time of test, 8-14 yrs	4/4	4/4
	Acquired Disturbance of vision first noted at age 15 to 57 yrs. Age at test, 16 to 59 yrs	5/6	—
Mothers who gave birth to hydrochephalic or microcephalic infants stillborn or died soon after birth		—	3/4
Mothers who gave birth to anencephalic monsters		—	3/8

*8 of the 10 individuals tested had the antibodies in their serum.

among 8 children with hydrocephalus and 2 infants with microcephaly but without either cerebral calcification or chorioretinitis in the macular region there was none with neutralizing antibodies. Similarly among 9 infants and children with histories of convulsions beginning soon after birth, psychomotor retardation, or palsies *not* associated with cerebral calcification or chorioretinitis there was but one child with neutralizing antibodies which were also present in the mother's serum. This child had convulsions and fever for several days after birth and subsequently there was some question of retardation of psychomotor development. It would appear, therefore, that, with one exception, the infants and children ranging in age from several months to 15 years, who together with their mothers had toxoplasma neutralizing antibodies, exhibited either *chorioretinitis in the macular region* or *cerebral calcification* or both in addition to the various other manifestations. It may be of interest to note here that on one occasion the serum of the father (1 of 4 tested) and older normal siblings have also been found to contain toxoplasma neutralizing antibodies, suggesting a high incidence of associated familial, mild or subclinical infection.

The finding of toxoplasma neutralizing antibodies in 3 of 4 mothers who had given birth to hydrocephalic or microcephalic infants, who were either stillborn or died soon after birth, was not surprising although this does not necessarily indicate the true incidence of toxoplasmic infection as the causative factor in such conditions. The possibility that toxoplasmic infection early enough in pregnancy might occasionally lead to anencephaly led to a study of the sera of 8 mothers who had given birth to anencephalic monsters. Three of the 8 had neutralizing antibodies for toxoplasma, negative Wassermanns and no hydramnios, 2 had positive Wassermanns and no hydramnios, and the remaining 3 had hydramnios. While this

evidence is suggestive, it cannot be taken as proof that toxoplasmic infection of the fetus may give rise to anencephaly, but further studies are indicated especially on the anencephalic monsters themselves.

Perhaps the most significant new lead, arising from the application of the neutralization test to various morbid conditions in which the pathogenic potentialities of toxoplasma might have been considered, was found in the high incidence of positive tests (9 of 10) among older children and adults presenting a chorioretinitis of unknown etiology resembling (particularly in its predilection for the macular region) that found in infants with proved congenital toxoplasmosis. The possibility that in at least some of these individuals, the chorioretinitis may have been the only important residue of a congenital toxoplasmosis which was not detected until the children had to begin to read, is suggested by the fact that the sera of the mothers of all 4 of these patients had neutralizing antibodies. In at least one family the sera of 2 older, normal siblings had no such antibody. On the other hand, the appearance of poor vision in later life in the other patients suggests an acquired infection.

Among the other individuals whose sera neutralized toxoplasma there were 2 children who in the summer of 1939, at 10 and 12 years of age respectively, had acute encephalitis characterized chiefly by fever, coma, and convulsions; one recovered completely and the other was left with a changed personality and persistent convulsions. There were also 5 individuals, 10 to 69 years of age, with unexplained encephalopathies presenting predominantly severe, persistent headaches, in some associated with hyperostosis frontalis interna. The sera of 2 laboratory workers exposed to toxoplasma were positive. The serum of a 14-year-old boy with an atypical pneumonia from which he recovered in one week following sulfathiazole therapy was in the positive group as were those of several rabbit handlers one of whom also developed agglutinins for *B. tularense*. Although the present data do not permit a final statement regarding the incidence of toxoplasma neutralizing antibodies among various age groups of the population at large, it would appear to be in the range of 10% or less if one excludes the members of the immediate family of individuals with clinical manifestations of toxoplasmosis.

Summary. A standardized procedure for carrying out the toxoplasma neutralization test in rabbits was described. The sera of 59 of 151 selected individuals were positive. A very high incidence of positive tests was found in infants and children presenting psychomotor disturbances with or without hydrocephalus or microcephaly only when these were associated with cerebral calcification or chorioretinitis in the macular region or both. The congenital character of the disease was confirmed by the regular presence of these antibodies in the mothers of the affected children. Chorioretinitis affecting predominantly the macular region, and resembling that seen in cases of proved congenital toxoplasmosis, but occurring in otherwise apparently normal children and adults was associated with neutralizing antibodies for toxoplasma in 9 of 10 individuals. Certain other morbid conditions associated with toxoplasma neutralizing antibodies were also briefly described.

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