J. Hyg., Camb. (1966), **64**, 347
With 1 *plate*
Printed in Great Britain

The survival of *Toxoplasma* in infected mosquitoes

By JENNIFER M. CHAPMAN and W. E. ORMEROD

Department of Parasitology,
London School of Hygiene and Tropical Medicine,
Keppel Street, London, W.C. 1

(*Received* 7 *March* 1966)

INTRODUCTION

It has often been suggested that arthropods can act as transmitting agents for *Toxoplasma gondii* Nicolle & Manceaux, and the evidence has been reviewed by Jacobs (1953), with further experiments by Laarman (1956), Rifaat & Morsy (1962), Deane (1958) and Nussenzweig & Deane (1958).

The experiments described in this paper were suggested by the following circumstantial evidence that the mosquito *Culiseta annulata* Shrank might be acting as the transmitting agent for *Toxoplasma* infection from pigs to man in and around the town of Barton-upon-Humber, Lincolnshire, England. The Medical Officer of Health had noted the persistently high perinatal mortality for this area for the years 1956–59 (Robertson, 1960) and had suggested that this might be due to toxoplasmosis on account of the unusually high rate of positive reactions to the *Toxoplasma* dye test of Sabin & Feldman (1948). He had also noted that in the 2–4 years age group the conversion from positive to negative occurred during the late summer; this, he believed, might indicate a mosquito-borne infection since the population of mosquitoes was abnormally high.

In October 1961 one of us (W.E.O.) visited Barton-upon-Humber together with Mr P. G. Shute and Miss Maryon of the Malaria Reference Laboratory. Even at this late season very large numbers of larvae of *C. annulata* were found in flooded claypits, the water of which had been contaminated with town rubbish giving the high organic content required by this species.

Pig production is an important industry in the area both on intensive and backyard scales; local veterinarians had diagnosed toxoplasmosis in pigs and this had been confirmed by the dye test.

There were many piggeries both in and around the town from which the following mosquitoes, of species known to bite man, were collected: 54 *Culiseta annulata*, 21 *Anopheles claviger* Meigen, 1 *A. labranchiae atroparvus* van Thiel.

These were ground up in batches of one to nine mosquitoes and injected into multimammate mice, *Mastomys coucha*, which were killed 5 weeks later and dye tests performed by Dr Fleck of Swansea. Experimental and control animals showed titres up to 1/8, but one experimental *Mastomys* into which seven *Culiseta* from a piggery in Barton-upon-Humber had been injected, showed a titre of 1/128. Despite prolonged searching of sections of the brain of this *Mastomys* no *Toxoplasma* cysts or free forms were seen.

The piggery in question had been abandoned shortly after the experiment because of unexplained losses and lack of fertility in the stock.

Next year ecological conditions had changed. As a result of late frosts, the massive growth of population of *Culiseta* that had appeared in previous years did not occur and a repetition of the abortive field experiments described above was not feasible. We therefore decided to investigate under laboratory conditions the fate of *Toxoplasma* free forms and cysts in *Culiseta annulata* and other mosquitoes, and in this way to obtain information on the susceptibility of mosquitoes to infection and their possible ability to act as transmitting agents for *Toxoplasma* in the field.

MATERIALS AND METHODS

Strain of Toxoplasma

Most previous experiments on the transmission of *Toxoplasma* by arthropods have been conducted using the acute RH strain of the organism. It seemed, however, unlikely that this type of strain would be involved in the relatively benign infections which occurred at Barton-upon-Humber. In the present series of experiments, therefore, we used an avirulent or chronic strain previously isolated from sheep during a survey of the East Midlands and Yorkshire (Beverley & Mackay, 1962; Beverley & Watson, 1961), kindly supplied to us by Dr Beverley. A chronic strain such as this, which on passage goes through more of its life-cycle, seems more likely to be transmissible by a vector than the RH strain which has undergone serial passage for many years involving only the trophozoite form.

Mice infected intraperitoneally with cysts of the Beverley strain produce only a slight peritoneal exudate; a few trophozoites can be seen in Giesma stained preparations from peritoneal fluid during the first 14 days but after this time they disappear and cannot be demonstrated by direct microscopy. This phase of multiplication is usually accompanied by a low level of parasitaemia. Remington, Melton & Jacobs (1961) showed that this was of short duration and was followed by localization and encystment in the brain and other organs. Cysts are detectable in the homogenate of brain material from about 14 days after infection.

The majority of animals do not show symptoms of disease but about 5% succumb. In these, 10 days after infection, the abdomen is distended with peritoneal exudate containing intra- and extracellular trophozoites.

For routine passage, 20 g. white mice obtained from Messrs Tucks each received an inoculum of approximately 20 cysts by the intraperitoneal route. Counting was performed on 0·01 ml. brain homogenate under a coverslip using phase contrast at $\times 100$ magnification.

The time interval between successive passages was about 3 months.

Production of maximum numbers of trophozoites

Mastomys were inoculated intraperitoneally with 0·01 ml. of a suspension of hydrocortisone acetate containing 250 mg./ml. (Hydrocortistab, Boots), 5 days before infection with about 300 cysts of *Toxoplasma*. On the 7th-8th day after infection there were at least six intracellular trophozoites per ml. On the ninth day the animals died with characteristic symptoms.

Production of the maximum numbers of cyst forms

Mice (15 g.) were inoculated intraperitoneally each with about 150 cysts. This resulted in loss of about 40% of the mice between the fifth and eleventh day following infection, and the remainder failed to develop in weight and size as rapidly as control mice of the same age. Brains of these infected mice examined from the tenth day onwards showed large numbers of cysts, which were small at first, ranging from about 7 to 10 μ in diameter and containing only a few organisms, and reached about 40 μ in diameter at 4 weeks. In these mice the concentration of cysts varied between 30 and 40 per 0·01 ml. sample of a homogenate of the whole brain in 1 ml. of saline.

It was also found that passage of the Beverley strain from the brains of infected laboratory mice into cotton rats (*Sigmodon*) increased the yield of cysts obtained on repassage to laboratory mice. The concentration from rat brain homogenate was found to be two to three cysts per 0·01 ml., and when this was repassaged intraperitoneally into 15 g. mice at the rate of 70–80 cysts per mouse, a final concentration of 40–50 cysts per 0·01 ml. sample of brain homogenate was obtained with similar mortality among the mice. This is equivalent to about double the yield of cysts using laboratory mice only.

Strains of mosquitoes

Experiments were carried out using four species of mosquitoes. Laboratory cultures of *Aedes aegypti*, *A. togoi* and *Anopheles labranchiae atroparvus* were available in the Department of Entomology at this School. A culture of *C. annulata* was established for use in these experiments from a collection of gravid females made at Barton-upon-Humber. Multiplication of the colony depended upon the use of artificial insemination.

Infection of mosquitoes and subsequent maintenance

The mosquitoes were fed on infected material through a membrane consisting of the skin of a freshly killed mouse, which was stretched over the bottom of a glass tube, 1·8 cm. in diameter. The bottom of the tube had been pressed in to form a concavity which contained the infective or control material on which the mosquitoes were to be fed. The tube contained water at 37° C., heated and made to circulate with a 'Circotherm' unit. The mouse skin rested on the top of a 4·5 in. cubical cage containing about 50 experimental mosquitoes. The apparatus was set up at air temperature of 20° C. for *C. annulata* and 25–26° C. for the other species.

Feeding was allowed to continue for about 1 hr., after which the fed females were separated by placing the cage in a totally enclosed transparent cabinet and transferring them by mechanical suction to another similar cage. The apparatus was constructed in such a way that there was no possibility of an infected mosquito escaping into the laboratory.

Two or three mosquitoes from each cage were ground up in saline and injected with 20,000 units of penicillin into mice daily for about 14 days. In some instances mosquitoes were fixed in Carnoy's solution, 'jellified' in benzene and embedded in

wax for sectioning. Sections of 4 μ were stained by the Giemsa-Colophonium method of Shortt & Cooper (1948). Homogenates of the brains of injected mice were examined after 1 month and if no cysts were observed, a further passage was made and the brain homogenate re-examined after another month. Stained smears and sections were also examined; they were fixed in Bouin's fluid and studied by the method of Lainson (1955).

The viability of toxoplasms in the feed was tested at the end of each feeding period by injection into mice.

In most experiments (see Tables 1 and 2) blood was added to the infective material. This was to ensure the passage of the infective material into the insect's stomach. In some instances blood was omitted, to test the survival of toxoplasms in the insect crop where the feed is partially retained in the absence of blood, as it was thought that toxoplasms remaining in the crop of a mosquito and not subjected to the digestive juices of the stomach might remain infective for longer.

In addition to the experiments carried out by membrane feeding *C. annulata* was also induced to feed directly on a mixture of homogenized brain and 10% sucrose. Controls showed that viability was not affected by this treatment. Since regurgitation from the crop under these conditions might be expected to occur, the mosquitoes were allowed to feed on baby mice which were examined for cysts in the brain after 1 month.

RESULTS

Results of experiments to infect mosquitoes with Toxoplasma *and of attempts to transmit the disease*

The survival of trophozoites in four species of mosquito which had been fed on infected material through a membrane was shown by inoculation of ground-up mosquitoes into mice to vary within narrow limits (see Table 1). Counting the day of feeding as the first day, viable trophozoites were present only on the first day in *Aedes togoi* and *Anopheles labranchiae atroparvus*, up to the second day in *Culiseta annulata*, and up to the third day in *Aedes aegypti*. These figures suggest that trophozoites remain viable as long as the blood meal remains undigested, and also that the different temperatures at which the cultures of fed mosquitoes were held did not affect their survival time.

Microscopic examination of the Giemsa-stained smears and sections of brain material of recipient mice failed to reveal cases of the infection which had not already been detected by examination of fresh material.

A number of cyst-like bodies were seen from time to time in fresh preparations, but because of the slight difference in their structure from true cyts of *Toxoplasma*, their random appearance in both control and experimental animals, their appearance only in fresh, never in stained preparations, and their failure to produce infection on inoculation into clean mice, we conclude that they were unrelated to *Toxoplasma*. (Similar bodies were observed by Dr Om Prakash while working in this laboratory.) One such body, found in brain material taken from a mouse inoculated with a number of ground-up *Aedes aegypti* is shown in Plate I. This was at first thought to be a cyst of *Toxoplasma*, as it was of a comparable size, but no

Table 1. *Summary of results of experiments to infect mosquitoes with trophozoite-forms of* Toxoplasma

Species of mosquito	No. of times experiment repeated	Presence of other fluid 1:1 ratio	Temperature of mosquito culture °C	Control	Interval (days) between infection of mosquito and injection of mosquito suspension into mouse, and presence or absence of cysts in the brain of that mouse 1 month later													
					1	2	3	4	5	6	7	8	9	10	11	12	13	14
Aedes aegypti	5	Blood	26	+	+	+	−	−	−	−	−	−	−	−	−	−	−	− 1st passage
			37		+	+	+	−	−	−	−	−	−	−	−	−	−	− 1st passage
			(26)		+	+	+	−	−	−	−	−	−	−	−	−	−	− 2nd passage
			(37)		+	+	+	−	−	−	−	−	−	−	−	−	−	− 2nd passage
Aedes togoi	2	Blood	26	+	+	−	−	−	−	−	−	−	−	−	−	−	−	− 1st passage
			37		+	−	−	−	−	−	−	−	−	−	−	−	−	− 1st passage
			(26)		+	−	−	−	−	−	−	−	−	−	−	−	−	− 2nd passage
			(37)		+	−	−	−	−	−	−	−	−	−	−	−	−	− 2nd passage
Anopheles l. atroparvus	4	Blood	26	+	+	+	−	−	−	−	−	−	−	−	−	−	−	− 1st passage
			37		+	+	−	−	−	−	−	−	−	−	−	−	−	− 1st passage
			(26)		+	+	−	−	−	−	−	−	−	−	−	−	−	− 2nd passage
			(37)		+	+	−	−	−	−	−	−	−	−	−	−	−	− 2nd passage
Anopheles l. atroparvus	1	—	26	+	+	+	−	−	−	−	−	−	−	−	−	−	−	− 1st passage
			37		+	+	−	−	−	−	−	−	−	−	−	−	−	− 1st passage
Culiseta annulata	3	Blood	20	+	+	+	−	−	−	−	−	−	−	−	−	−	−	− 1st passage
					+	+	−	−	−	−	−	−	−	−	−	−	−	− 1st passage
					+	+	−	−	−	−	−	−	−	−	−	−	−	− 2nd passage
					+	+	−	−	−	−	−	−	−	−	−	−	−	− 2nd passage

In several cases mice died before examination for cysts was possible. These deaths are not recorded in the table, as parallel experiments covered such instances

Key: +, Cysts found in mouse brain when examined microscopically 1 month after inoculation with mosquitoes. −, No cysts found. Control: the viability of toxoplasms in the feed at the time of experiment was tested by inoculating mice with a sample of the feed.

Table 2. *Summary of results of experiments to infect* Culiseta annulata *with cyst-forms of* Toxoplasma gondii

Species of mosquito	No. of times experiment repeated	Presence of other fluid 1:1 ratio	Temperature of mosquito culture °C	Control	Interval (days) between infection of mosquito and injection of mosquito suspension into mouse, and presence or absence of cysts in the brain of that mouse 1 month later														
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	
C. annulata	3	Blood	20	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	1st passage
			26		+	+	−	−	−	−	−	−	−	−	−	−	−	−	
					+	+	−	−	−	−	−	−	−	−	−	−	−	−	2nd passage
					+	+	−	−	−	−	−	−	−	−	−	−	−	−	
C. annulata	3	10 % sucrose	20	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	1st passage
					+	+	+	−	−	−	−	−	−	−	−	−	−	−	2nd passage

Key: +, Cysts found in mouse brain, when examined microscopically, 1 month after inoculation with mosquitoes, or 1st passage mouse brain. −, No cysts found. Control: the viability of toxoplasms in the feed at the time of experiment was tested by inoculating mice with a sample of the feed.

disease was produced in two passages of this material through mice. As experience was obtained it became apparent that the wall of this 'cyst' was penetrated by several pores, and that the 'trophozoites' had a honeycomb appearance which became particularly obvious in partly dried preparations, as in Plate 1, fig. 2. Although we were uncertain of the nature of this body it seemed unlikely to be a parasitic protozoon.

Stained sections of mosquitoes, when examined under the microscope, did not reveal any sign of parasites, possibly because it was often difficult to obtain a good section of the blood meal within the mosquito stomach.

The experiment in which *A. l. atroparvus* were fed on a blood-free meal of infective trophozoites and then injected into mice showed that infective *Toxoplasma* were retained up to the second day only.

There was no evidence of transmission of the disease to baby mice on which infected mosquitoes had been allowed to feed.

Results of experiments to infect Culiseta annulata *with cyst-forms of* Toxoplasma gondii, *and to determine parasite survival time*

The survival of viable *Toxoplasma* in *C. annulata* fed on infected brain containing cysts and 10% sucrose was shown, by mouse inoculation, to last up to the third day, but when mixed with blood the parasites remained viable only up to the second day (Table 2).

All attempts to transmit the infection to anaesthetized baby mice, by allowing artificially infected mosquitoes to feed on them, were unsuccessful.

Careful examination of Giemsa stained crush-smears of crops dissected from mosquitoes which had fed on the infected brain-sucrose mixtures showed no cysts.

DISCUSSION

In the inconclusive field study which suggested the experiments recorded in this paper, it appeared that the *Culiseta annulata*, which was present in exceptionally large numbers at Barton-upon-Humber, might be connected with the high rate of dye-test antibodies in man; we therefore wished to investigate the possibility that *Toxoplasma* taken up in the mosquitoes' feed might be transmissible to another host. We found that the low roofs of pigsties where the mosquitoes rested were often very hot and we felt that this might be a factor in making the mosquitoes infective. In fact we could not demonstrate any effect of temperature, although this may have been because the mosquitoes did not live long enough under the experimental conditions.

Mosquitoes might become infected in the field in two ways: by taking trophozoites in blood or lymph from an acutely infected animal or by taking up cysts while probing the subcutaneous tissue of an animal harbouring a chronic infection, and the organisms obtained in either way might lodge in the crop or in the stomach of the mosquito. Although it is most unlikely that under natural conditions as high a concentration of organisms could be obtained in the infective feed as we achieved in our experiments, yet some degree of concentration of organisms might result by

repeated feeding on infected animals. The ability of a mosquito under these conditions to become an efficient transmitting agent presupposes that the infective organism develops in it and this we have been unable to demonstrate.

We have, however, demonstrated conclusively that a mosquito can retain an infection with cysts or trophozoites up to the third day from the time of infection and that it is not infective after this. We cannot exclude the possibility that an occult form is present and that it becomes infective after the fourteenth day.

We do not think that transient infectiveness of mosquitoes is likely to be a major contribution to the spread of toxoplasmosis, the more since the bite of mosquitoes during this period has not been shown to be infective; especially since the survival time of the organism is less than the normal interval between successive feeds in nature. It is necessary either to inject the mosquito or at least to crush it into a wound to obtain transmission. Yet, despite these limitations, so frequently is the organism found in nature that it is not unlikely that infection in man can occasionally occur by this means, i.e. by the crushing of an infected mosquito into a skin abrasion.

SUMMARY

1. An incomplete field study in Lincolnshire, England, suggested that toxoplasmosis might be transmitted from pig to man by mosquitoes. Although the results were inconclusive they suggested the experimental work recorded in this paper.

2. Trophozoites of the Beverley strain were obtained in increased yield from the peritoneal fluid of *Mastomys* treated with hydrocortisone. Cysts were obtained in increased yield by passage of the brain of *Sigmodon* into laboratory mice.

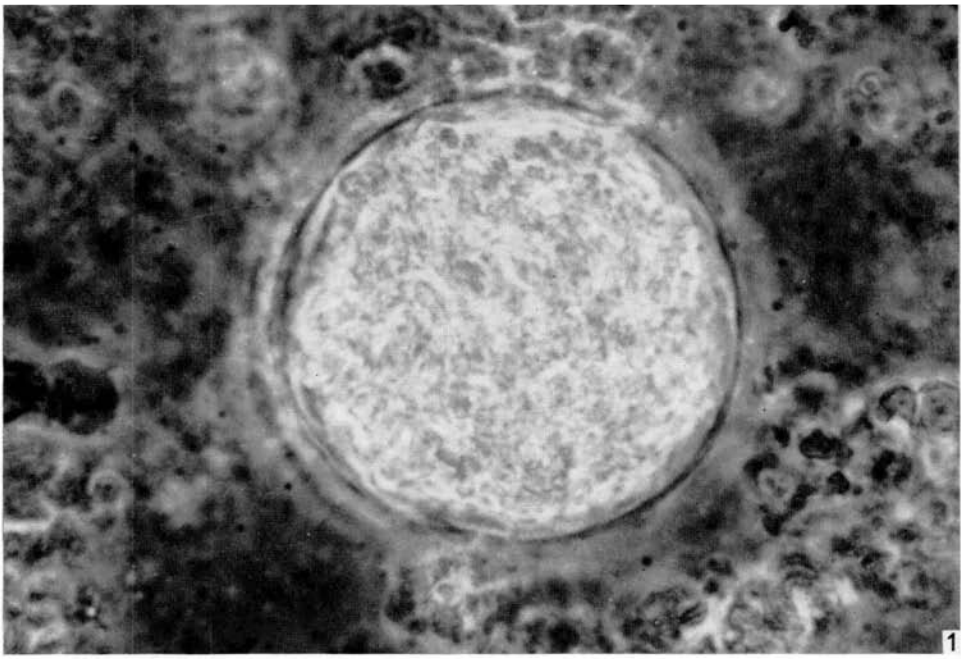
3. Four species of mosquitoes were fed through a membrane or on sucrose solution on media containing either cysts or trophozoites of *Toxoplasma gondii*. In some experiments blood was added to ensure passage of the feed to the mosquito stomach, in others it was excluded so that at least part would pass to the crop.

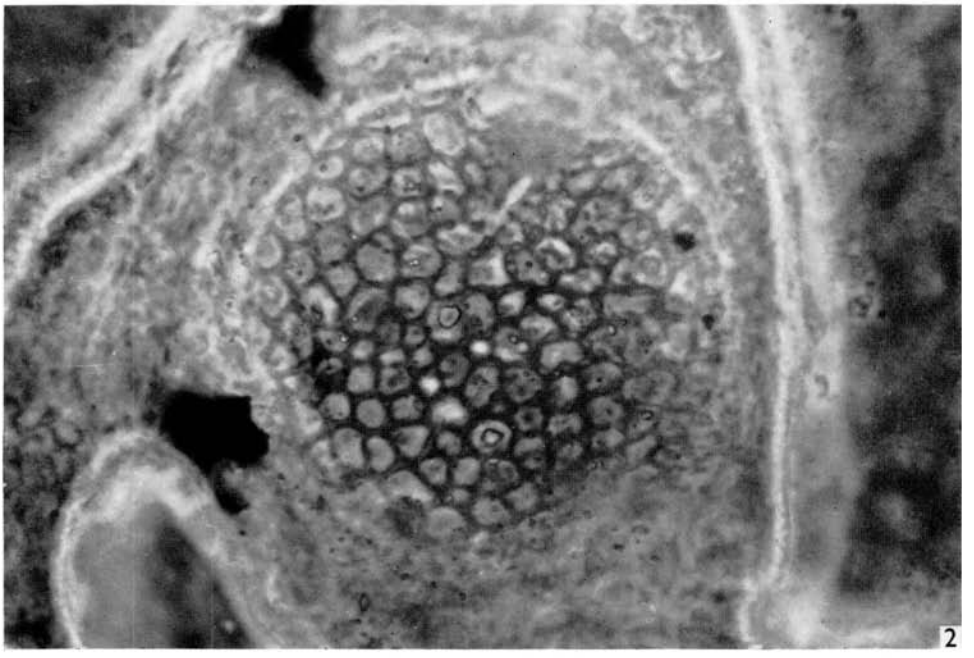
4. A spurious 'cyst' resembling but distinguishable from *Toxoplasma* was noted in the homogenate of mouse brain.

5. In no instance did mosquitoes retain infectivity as demonstrated by injection into mice, beyond the third day, but since experiments were not carried on beyond the fourteenth day the existence of an occult form cannot be excluded.

We would like to thank Dr J. S. Robertson, Prof. D. S. Bertram and Prof. P. C. C. Garnham for advice and practical help; also Mr M. J. Page for technical assistance.

The work was financed by the Medical Research Council.





https://doi.org/10.1017/S0022172400040626 Published online by Cambridge University Press

REFERENCES

BEVERLEY, J. K. A. & MACKAY, R. R. (1962). Ovine abortion and toxoplasmosis in the East Midlands. *Vet. Rec.* **74**, 499.

BEVERLEY, J. K. A. & WATSON, W. A. (1961). Ovine abortion and toxoplasmosis in Yorkshire. *Vet. Rec.* **73**, 6.

DEANE, M. P. (1958). Estudos sobre transmissao do *Toxoplasma gondii*. II. Nota sobre a transmissao experimental pelo carrapato *Amblyomma cajennense*. *Revta bras. Malar. Doenç. trop.* **10**, 551.

JACOBS, L. (1953). The biology of *Toxoplasma*. *Am. J. trop. Med. Hyg.* **2**, 365.

LAARMAN, J. J. (1956). Transmission of experimental toxoplasmosis by *Stomoxys calcitrans*. *Documenta Med. geogr. trop.* **8**, 293.

LAINSON, R. (1955). Studies on the epidemiology of *Toxoplasma gondii* in England. The study of *Toxoplasma* in fixed and stained preparations. University of London Ph.D. Thesis vol. 1, p. 149).

NUSSENZWEIG, R. S. & DEANE, M. P. (1958). Estudos sobre transmissao do *Toxoplasma gondii*. I. Experiencias com triatomineos. *Revta bras. Malar. Doenç. trop.* **10**, 543.

REMINGTON, J. S., MELTON, M. L. & JACOBS, L. (1961). Induced and spontaneous recurrent parasitemia in chronic infections with avirulant strains of *Toxoplasma gondii*. *J. Immun.* **87**, 578.

RIFAAT, M. A. & MORSY, T. A. (1962). Experimental transmission of toxoplasmosis in laboratory animals and birds. *J. trop. Med. Hyg.* **65**, 314.

ROBERTSON, J. S. (1960). Excessive perinatal mortality in a small town associated with evidence of toxoplasmosis. *Br. med. J.* ii, 91.

SABIN, A. B. & FELDMAN, H. A. (1948). Dyes as microchemical indicators of a new immunity phenomenon affecting a protozoon parasite (Toxoplasma). *Science, N.Y.*, **108**, 660.

SHORTT, H. E. & COOPER, W. (1948). Staining of microscopical sections containing protozoal parasites by modifications of McNamara's method. *Trans. R. Soc. trop. Med. Hyg.* **41**, 427.

EXPLANATION OF PLATE

Comparison of spurious 'toxoplasma cyst' with a cyst of *Toxoplasma gondii* of comparable size (60 μ across), both photographed under phase contrast with × 100 oil-immersion objective, from the homogenate of mouse brain.

Fig. 1. Cyst of *Toxoplasma* showing unbroken cyst wall and the pattern produced by internal trophozoites.

Fig. 2. Spurious 'cyst' showing honeycomb pattern (exaggerated by the preparation being partly dried).